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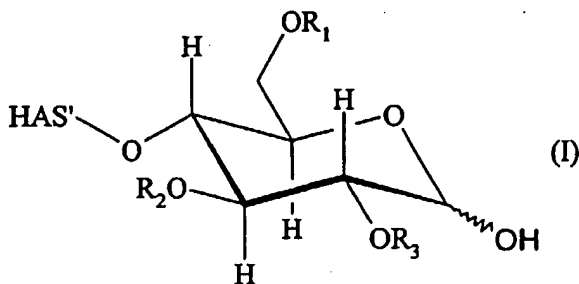
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WO 2004/024777 A1

(54) Title: **HYDROXYALKYL STARCH DERIVATIVES**



hydroxyalkyl starch derivative.

(57) Abstract: The present invention relates to a method of producing a hydroxyalkyl starch derivative comprising reacting hydroxyalkyl starch of formula (I) at its reducing end which is not oxidized prior to said reaction, with a compound of formula (II) R'NH-R'' (II) wherein R<sub>1</sub>-R<sub>2</sub> and R<sub>3</sub> are independently hydrogen or a linear or branched hydroxyalkyl group, and wherein either R' or R'' or R' and R'' comprise at least one functional group X capable of being reacted with at least one other compound prior to or after the reaction of (I) and (II), as well as to the hydroxyalkyl starch derivative as such, obtainable by said method, and to a pharmaceutical composition comprising said hy-

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### Hydroxyalkyl Starch Derivatives

The present invention relates to hydroxyalkyl starch derivatives, particularly hydroxyalkyl starch derivatives obtainable by a process in which hydroxyalkyl starch is reacted with a primary or secondary amino group of a linker compound. According to an especially preferred embodiment, the present invention relates to hydroxyalkyl starch derivatives obtainable by a process according to which hydroxyalkyl starch is reacted with a primary or secondary amino group of a linker compound and the resulting reaction product is reacted with a polypeptide, preferably with a glycoprotein and especially preferably with erythropoietin, via at least one other reactive group of the linker compound. A hydroxyalkyl starch which is especially preferred is hydroxyethyl starch. According to the present invention, the hydroxyalkyl starch and preferably the hydroxyl ethyl starch is reacted with the linker compound at its reducing end which is not oxidized prior to said reaction.

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Hydroxyethyl starch (HES) is a derivative of naturally occurring amylopectin and is degraded by alpha-amylase in the body. HES is a substituted derivative of the carbohydrate polymer amylopectin, which is present in corn starch at a concentration of up to 95 % by weight. HES exhibits advantageous biological properties and is used as a blood volume replacement agent and in hemodilution therapy in the clinics (Sommermeier et al., 1987, Krankenhauspharmazie, 8(8), 271-278; and Weidler et al., 1991, Arzneimittel-Forschung/Drug Res., 41, 494-498).

25

Amylopectin consists of glucose moieties, wherein in the main chain alpha-1,4-glycosidic bonds are present and at the branching sites alpha-1,6-glycosidic bonds are found. The physical-chemical properties of this molecule are mainly determined by the type of glycosidic bonds. Due to the nicked alpha-1,4-glycosidic bond, helical structures with about six glucose-monomers per turn are produced. The physico-

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chemical as well as the biochemical properties of the polymer can be modified via substitution. The introduction of a hydroxyethyl group can be achieved via alkaline hydroxyethylation. By adapting the reaction conditions it is possible to exploit the different reactivity of the respective hydroxy group in the unsubstituted glucose  
5 monomer with respect to a hydroxyethylation. Owing to this fact, the skilled person is able to influence the substitution pattern to a limited extent.

Some ways of producing a hydroxyethyl starch derivative are described in the art.

10 DE 26 16 086 discloses the conjugation of hemoglobin to hydroxyethyl starch wherein, in a first step, a cross-linking agent, e.g. bromocyanide, is bound to hydroxyethyl starch and subsequently hemoglobin is linked to the intermediate product.

One important field in which HES is used is the stabilisation of polypeptides which  
15 are applied, e.g., to the circulatory system in order to obtain a particular physiological effect. One specific example of these polypeptides is erythropoietin, an acid glycoprotein of approximately 34,000 kD which is essential in regulating the level of red blood cells in the circulation.

20 A well-known problem with the application of polypeptides and enzymes is that these proteins often exhibit an unsatisfactory stability. Especially erythropoietin has a relatively short plasma half life (Spivak and Hogans, 1989, Blood 73, 90; McMahon et al., 1990, Blood 76, 1718). This means that therapeutic plasma levels are rapidly lost and repeated intravenous administrations must be carried out. Furthermore, in  
25 certain circumstances an immune response against the peptides is observed.

It is generally accepted that the stability of polypeptides can be improved and the immune response against these polypeptides is reduced when the polypeptides are coupled to polymeric molecules. WO 94/28024 discloses that physiologically active  
30 polypeptides modified with polyethyleneglycol (PEG) exhibit reduced immunogenicity and antigenicity and circulate in the bloodstream considerably longer than unconjugated proteins, i.e. have a longer clearance rate. However, PEG-drug conjugates

exhibit several disadvantages, e.g. they do not exhibit a natural structure which can be recognized by elements of in vivo degradation pathways. Therefore, apart from PEG-conjugates, other conjugates and protein polymerates have been produced. A plurality of methods for the cross-linking of different proteins and macromolecules such as polymerase have been described in the literature (see e.g. Wong, Chemistry of protein conjugation and cross-linking, 1993, CRCs, Inc.).

The HES-drug conjugates disclosed in the art suffer from the disadvantage that HES is not conjugated site-specifically to the drug. Consequently, the conjugation results in a very heterogenous product having many components that may be inactive due to the destruction of the 3-dimensional structure during the conjugation step. Therefore, there is a need for further improved HES-polypeptides conjugates with improved stability and/or bioactivity.

One method of producing these conjugates uses, as starting material, an oxidized form of HES which is reacted with a crosslinking compound wherein the resulting product is reacted with a polypeptide or further modified and subsequently reacted with a polypeptide. It is a major disadvantage of this method that in a first step, the original HES has to be selectively oxidized, generally at its reducing end, by oxidizing the terminal aldehyde group and/or hemiacetale group to a lactone, thus rendering the overall process more difficult and expensive.

WO 02/08079 A2 discloses compounds comprising a conjugate of an active agent and a hydroxyalkyl starch wherein active agent and hydroxyalkyl starch are either linked directly or via a linker compound. As far as the direct linkage is concerned, the reaction of active agent and hydroxyalkyl starch is carried out in an aqueous medium which comprises at least 10 wt.-% of water. No examples are given which are directed to a hydroxyalkyl starch derivative which is produced by reacting hydroxyalkyl starch at its reducing end with a crosslinking compound comprising the structure unit -NH- in an aqueous medium. All examples are directed to hydroxyalkyl starch which is oxidized prior to a further reaction, the specific teaching of WO 02/08079 A2 thus having the aforementioned disadvantages.

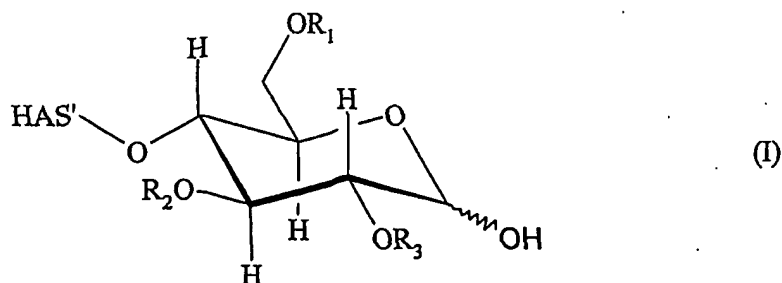
Therefore, it is an object of the present invention to provide a method of producing a hydroxyalkyl starch derivative which allows for reacting hydroxyalkyl starch at its reducing end with a suitable compound wherein the reducing end of the starch is not oxidized prior to the reaction.

It is a further object of the present invention to provide a method of producing a hydroxyalkyl starch derivative which allows for reacting hydroxyalkyl starch at its reducing end with a suitable compound wherein the reducing end of the starch is not oxidized prior to the reaction, said method being further characterized in that the reaction product of the reaction of hydroxyalkyl starch at its reducing end with a suitable compound is further reacted with at least one further compound.

It is a still further object of the present invention to provide a method as described above wherein the at least one further compound is a polypeptide, preferably a protein, more preferably erythropoietin.

It is yet another object of the present invention to provide a hydroxyalkyl starch derivative which is obtainable by a method as described above which comprises reacting hydroxyalkyl starch at its reducing end with a suitable compound wherein the reducing end of the starch is not oxidized prior to the reaction.

Therefore, the present invention relates to a method of producing a hydroxyalkyl starch derivative comprising reacting hydroxyalkyl starch (HAS) of formula (I)



at its reducing end which is not oxidized prior to said reaction, with a compound of formula (II)



wherein  $R_1$ ,  $R_2$  and  $R_3$  are independently hydrogen or a linear or branched hydroxyalkyl group, and wherein either  $R'$  or  $R''$  or  $R'$  and  $R''$  comprise at least one functional group X capable of being reacted with at least one other compound prior to or after the reaction of (I) and (II).

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In the context of the present invention, the term "hydroxyalkyl starch" (HAS) refers to a starch derivative which has been substituted by at least one hydroxyalkyl group. Therefore, the term hydroxyalkyl starch as used in the present invention is not limited to compounds where the terminal carbohydrate moiety comprises hydroxyalkyl groups  $R_1$ ,  $R_2$ , and/or  $R_3$  as depicted, for the sake of brevity, in formula (I), but also refers to compounds in which at least one hydroxy group present anywhere, either in the terminal carbohydrate moiety and/or in the remaining part of the starch molecule, HAS', is substituted by a hydroxyalkyl group  $R_1$ ,  $R_2$ , or  $R_3$ .

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15 In this context, the alkyl group may be a linear or branched alkyl group which may be suitably substituted. Preferably, the hydroxyalkyl group contains 1 to 10 carbon atoms, more preferably from 1 to 6 carbon atoms, more preferably from 1 to 4 carbon atoms, and even more preferably 2-4 carbon atoms. "Hydroxyalkyl starch" therefore preferably comprises hydroxyethyl starch, hydroxypropyl starch and hydroxybutyl starch, wherein hydroxyethyl starch and hydroxypropyl starch are particularly preferred.

20

Hydroxyalkyl starch comprising two or more different hydroxyalkyl groups are also possible.

25

The at least one hydroxyalkyl group comprised in HAS may contain two or more hydroxy groups. According to a preferred embodiment, the at least one hydroxyalkyl group comprised HAS contains one hydroxy group.

30

The expression "hydroxyalkyl starch" also includes derivatives wherein the alkyl group is mono- or polysubstituted. In this context, it is preferred that the alkyl group

is substituted with a halogen, especially fluorine, or with an aryl group, provided that the HAS remains soluble in water. Furthermore, the terminal hydroxy group of a hydroxyalkyl group may be esterified or etherified.

- 5 Furthermore, instead of alkyl, also linear or branched substituted or unsubstituted alkene groups may be used.

Hydroxyalkyl starch is an ether derivative of starch. Besides of said ether derivatives, also other starch derivatives can be used in the context of the present invention. For  
10 example, derivatives are useful which comprise esterified hydroxy groups. These derivatives may be e.g. derivatives of unsubstituted mono- or dicarboxylic acids with 2-12 carbon atoms or of substituted derivatives thereof. Especially useful are derivatives of unsubstituted monocarboxylic acids with 2-6 carbon atoms, especially derivatives of acetic acid. In this context, acetyl starch, butyl starch and propyl starch  
15 are preferred.

Furthermore, derivatives of unsubstituted dicarboxylic acids with 2-6 carbon atoms are preferred.

- 20 In the case of derivatives of dicarboxylic acids, it is useful that the second carboxy group of the dicarboxylic acid is also esterified. Furthermore, derivatives of monoalkyl esters of dicarboxylic acids are also suitable in the context of the present invention.

- 25 For the substituted mono- or dicarboxylic acids, the substitute groups may be preferably the same as mentioned above for substituted alkyl residues.

Techniques for the esterification of starch are known in the art (see e.g. Klemm D. et al, Comprehensive Cellulose Chemistry Vol. 2, 1998, Wiley-VCH, Weinheim, New  
30 York, especially chapter 4.4, Esterification of Cellulose (ISBN 3-527-29489-9).

Hydroxyethyl starch (HES) is most preferred for all embodiments of the present invention.

Therefore, the present invention also relates to a method as described above wherein  
5 the hydroxyalkyl starch is hydroxyethyl starch.

HES is mainly characterized by the molecular weight distribution and the degree of substitution. There are two possibilities of describing the substitution degree:

- 10 1. The substitution degree can be described relatively to the portion of substituted glucose monomers with respect to all glucose moieties (DS).
2. The substitution degree can be described as the "molar substitution" (MS), wherein the number of hydroxyethyl groups per glucose moiety are described.

15

HES solutions are present as polydisperse compositions, wherein each molecule differs from the other with respect to the polymerisation degree, the number and pattern of branching sites, and the substitution pattern. HES is therefore a mixture of compounds with different molecular weight. Consequently, a particular HES solution is  
20 determined by average molecular weight with the help of statistical means. In this context,  $M_n$  is calculated as the arithmetic mean depending on the number of molecules. Alternatively,  $M_w$ , the weight mean, represents a unit which depends on the mass of the HES.

25 In the context of the present invention, hydroxyethyl starch may have a mean molecular weight (weight mean) of from 1 to 300 kDa, wherein a mean molecular weight of from 5 to 100 kDa is more preferred. Hydroxyethyl starch can further exhibit a molar degree of substitution of from 0.1 to 0.8 and a ratio between  $C_2 : C_6$  substitution in the range of from 2 to 20 with respect to the hydroxyethyl groups.

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As far as the residues  $R_1$ ,  $R_2$  and  $R_3$  according to formula (I) are concerned there are no specific limitations given that compound (I) remains capable of being reacted



with a compound according to formula (II). According to a preferred embodiment, R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> are independently hydrogen or a hydroxyalkyl group, a hydroxyaryl group, a hydroxyaralkyl group or a hydroxyalkaryl group having of from 1 to 10 carbon atoms. Hydrogen and hydroxyalkyl groups having of from 1 to 6 carbon atoms  
5 are preferred. The alkyl, aryl, aralkyl and/or alkaryl group may be linear or branched and suitably substituted.

Therefore, the present invention also related to a method as described above wherein R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> are independently hydrogen or a linear or branched hydroxyalkyl  
10 group with from 1 to 6 carbon atoms.

Thus, R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> may be hydroxyhexyl, hydroxypentyl, hydroxybutyl, hydroxypropyl such as 1-hydroxypropyl, 2-hydroxypropyl, 3-hydroxypropyl, 1-hydroxyisopropyl, 2-hydroxyisopropyl, hydroxyethyl such as 1-hydroxyethyl, 2-  
15 hydroxyethyl, or hydroxymethyl. Hydrogen and hydroxyethyl groups are preferred, hydrogen and the 2-hydroxyethyl group being especially preferred.

Therefore, the present invention also relates to a method as described above wherein R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> are independently hydrogen or a 2-hydroxyethyl group.

20

According to the present invention, hydroxyalkyl starch is reacted with a compound of formula (II) wherein compound (II) may be reacted with another compound prior to the reaction with compound (I), to give a hydroxyalkyl starch derivative. As to compound (II), there are no specific limitations if compound (II) is capable of being  
25 reacted via the NH group bridging R' and R" with compound (I) at its reducing end which is not oxidized, to give a hydroxyalkyl starch derivative.

Preferred residues R' of compound (II) are hydrogen and alkyl, cycloalkyl, aryl, aralkyl, arylcycloalkyl, alkaryl or cycloalkylaryl residues where cycloalkyl, aryl, aralkyl, arylcycloalkyl, alkaryl or cycloalkylaryl residues may be linked directly to the NH  
30 group bridging R' and R" of compound (II) or, according to another embodiment, may be linked by an oxygen bridge to the NH group bridging R' and R" of compound

(II). The alkyl, aryl, aralkyl or alkaryl residues may be suitably substituted. As preferred substituents, halogenes such as F, Cl or Br may be mentioned. Especially preferred residues R' are hydrogen, alkyl and alkoxy groups, and even more preferred are hydrogen and unsubstituted alkyl and alkoxy groups.

5

Therefore, the present invention also relates to a method as described above wherein R' is hydrogen or a linear or branched alkyl or alkoxy group.

Among the alkyl and alkoxy groups, groups with 1, 2, 3, 4, 5, or 6 C atoms are preferred. More preferred are methyl, ethyl, propyl, isopropyl, methoxy, ethoxy, propoxy, and isopropoxy groups. Especially preferred are methyl, ethyl, methoxy, ethoxy, and particular preference is given to methyl or methoxy.

Therefore, the present invention also relates to a method as described above wherein R' is hydrogen or a methyl or a methoxy group.

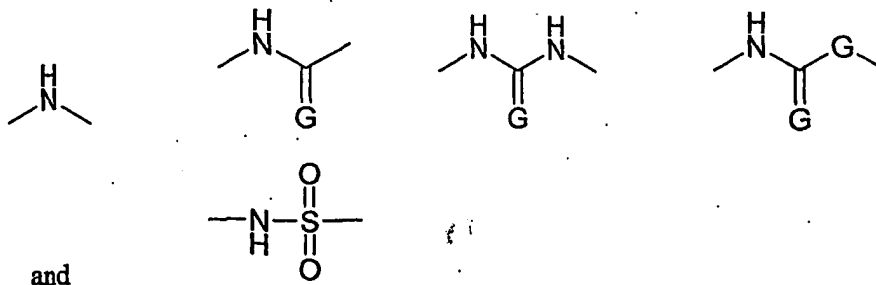
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Apart from the functional group X, R'' may comprise at least one additional functional group W. This at least one additional functional group W generally may be anywhere in R''. Preferably, W is directly linked to the NH group R' is linked to.

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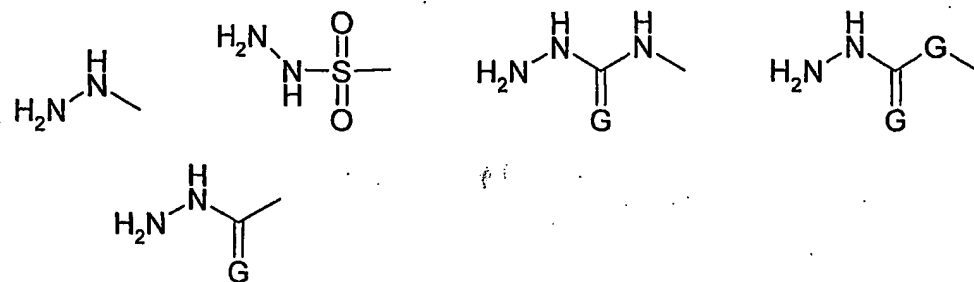
In general, there are no specific limitations regarding functional group W given that compound (I) is capable of being reacted with compound (II). In preferred embodiments, functional group W comprises the structure unit -NH- and/or the structure unit -(C=G)- where G is O or S, and/or the structure unit -SO<sub>2</sub>-. According to more preferred embodiments, the functional group W is selected from the group consisting of

25



where, if G is present twice, it is independently O or S.

According to preferred embodiments of the present invention where R' is H and W is linked directly to the NH group bridging R' and R'', R' and the NH group bridging R' and R'' form, together with W, the one of following groups:



5

As far as the at least one functional group X which is comprised in R' and/or R'', preferably in R'', no specific limitations exist. In general, all functional groups are possible which allow the reaction with at least one further compound.

- 10 As far as this reaction with a further compound is concerned, all kinds of interactions of the at least one functional group with the at least one further compound are possible. Among others, reactions of the at least one functional group X with a further compound are possible which lead to a covalent linkage, a ionic linkage and/or a van-der-Waals linkage, the covalent linkage being especially preferred.

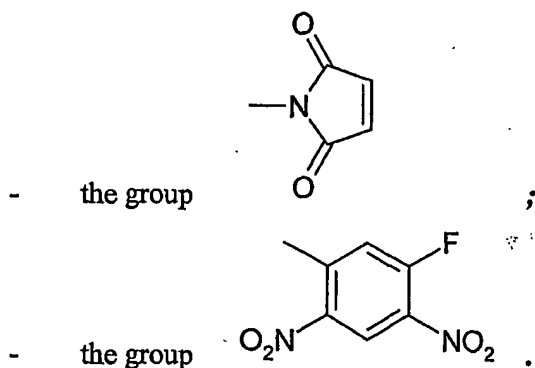
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Among others, the following functional groups X are to be mentioned:

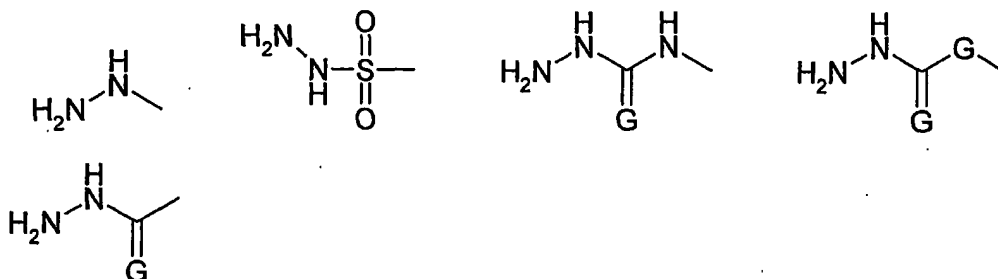
- C-C-double bonds or C-C-triple bonds or aromatic C-C-bonds;
- the thio group or the hydroxy groups;
- alkyl sulfonic acid hydrazide, aryl sulfonic acid hydrazide;
- 20 - 1,2-dioles;
- 1,2-aminoalcohols;
- the amino group  $-\text{NH}_2$  or derivatives of the amino groups comprising the structure unit  $-\text{NH}-$  such as aminoalkyl groups, aminoaryl group, aminoaralkyl groups, or alkarlyaminogroups;
- 25 - the hydroxylamino group  $-\text{O}-\text{NH}_2$ , or derivatives of the hydroxylamino group comprising the structure unit  $-\text{O}-\text{NH}-$ , such as hydroxylalkylamino groups, hy-

droxylaryl amino groups, hydroxylaralkyl amino groups, or hydroxalalkaryl amino groups;

- alkoxy amino groups, aryloxy amino groups, aralkyloxy amino groups, or alkaryloxy amino groups, each comprising the structure unit -NH-O-;
- 5 - residues having a carbonyl group, -Q-C(=G)-M, wherein G is O or S, and M is, for example,
  - -OH or -SH;
  - an alkoxy group, an aryloxy group, an aralkyloxy group, or an alkaryloxy group;
  - 10 -- an alkylthio group, an arylthio group, an aralkylthio group, or an alkarylthio group;
  - an alkylcarbonyloxy group, an arylcarbonyloxy group, an aralkylcarbonyloxy group, an alkarylcarbonyloxy group;
  - activated esters such as esters of hydroxylamines having imid structure
  - 15 such as N-hydroxysuccinimide or having a structure unit O-N where N is part of a heteroaryl compound or, with G = O and Q absent, such as aryloxy compounds with a substituted aryl residue such as pentafluorophenyl, paranitrophenyl or trichlorophenyl;
- wherein Q is absent or NH or a heteroatom such as S or O;
- 20 - -NH-NH<sub>2</sub>, or -NH-NH-;
- -NO<sub>2</sub>;
- the nitril group;
- carbonyl groups such as the aldehyde group or the keto group;
- the carboxy group;
- 25 - the -N=C=O group or the -N=C=S group;
- vinyl halide groups such as the vinyl iodide or the vinyl bromide group or vinyl triflate;
- -C≡C-H;
- -(C=NH<sub>2</sub>Cl)-OAlkyl
- 30 - groups -(C=O)-CH<sub>2</sub>-Hal wherein Hal is Cl, Br, or I;
- -CH=CH-SO<sub>2</sub>-;
- a disulfide group comprising the structure -S-S-;



Among these groups, the thio group, the amino group, the hydroxylamino group, the alkoxyamino groups and the following groups are especially preferred:



- 5 Therefore, the present invention also relates to a method as described above wherein the at least one functional group X is selected from the group consisting of -SH, -NH<sub>2</sub>, -O-NH<sub>2</sub>, -NH-O-alkyl, -(C=G)-NH-NH<sub>2</sub>, -G-(C=G)-NH-NH<sub>2</sub>, -NH-(C=G)-NH-NH<sub>2</sub>, and -SO<sub>2</sub>-NH-NH<sub>2</sub> where G is O or S and, if G is present twice, it is independently O or S.

10

As far as the alkoxyamino groups are concerned, particular preference is given to the propoxyamino group, the ethoxyamino group and the methoxyamino group, the methoxyamino group -NH-O-CH<sub>3</sub> being especially preferred.

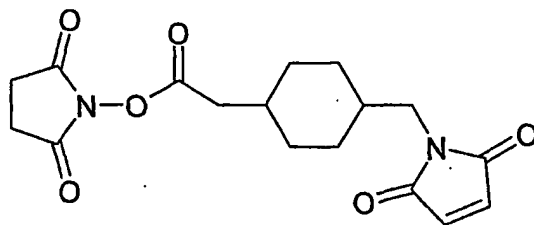
- 15 According to yet another aspect of the present invention, the at least one functional group X may be a group which is not capable of reacting directly with a given further compound but which may be chemically modified in order to be capable of reacting in the desired way. This modification of the functional group X comprised in compound (II) may be carried out either prior to the reaction of compound (II) with com-

5    pound (I) or after the reaction of compound (II) with compound (I). If compound (II) comprises at least two, optionally chemically different, functional groups X, it is possible to modify at least one functional group X prior to the reaction of compound (II) with compound (I) and at least one functional group X after the reaction of compound (II) with compound (I).

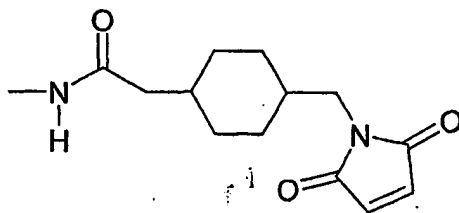
As an example of a functional group X to be modified prior to the reaction with a further compound, a 1,2-amino alcohol or a 1,2-diol may be mentioned which is modified, e.g., by oxidation to form an aldehyd or a keto group.

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Another example for a functional group X to be modified prior to the reaction with a further compound is a -NH<sub>2</sub> group which is modified by the reaction with, e.g., a compound according to the following formula

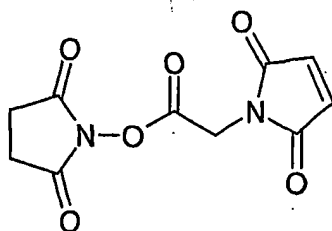


to give a structure of the following formula

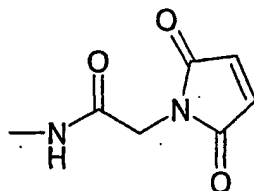


15    which is, e.g., reactive towards a thio group.

Another example for a functional group X to be modified prior to the reaction with a further compound is a -NH<sub>2</sub> group which is modified by the reaction with, e.g., a compound according to the following formula

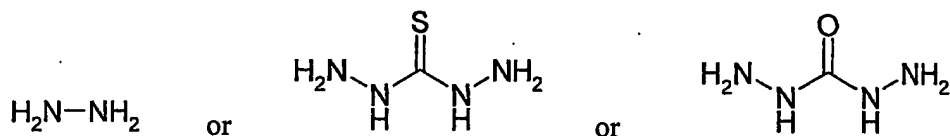


to give a structure of the following formula



which is, e.g., reactive towards a thio group.

The at least one functional group X may be linked directly to the NH group bridging  
 5 R' and R". Thus, according to one embodiment of the present invention, the functional group X is equivalent to R". Specific examples of compounds where X is directly linked to the NH group bridging R' and R" are, among others,

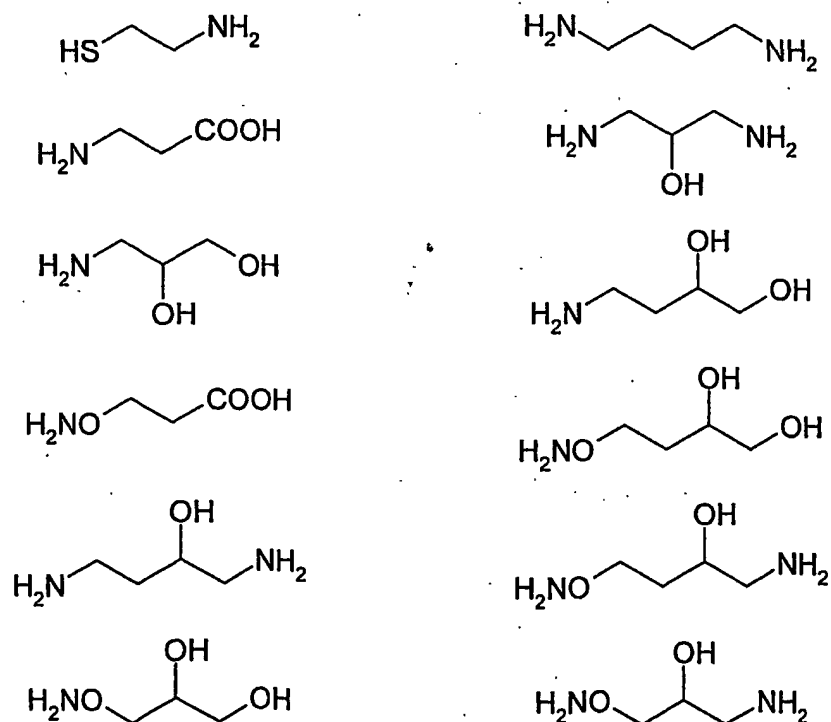


Another specific example of a such a compound which is also comprised in the present invention is NH<sub>3</sub>.  
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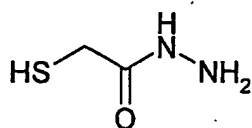
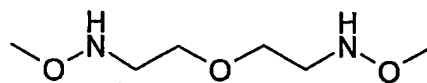
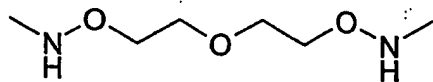
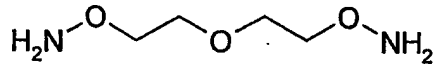
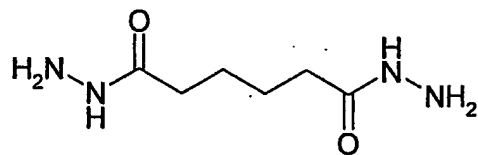
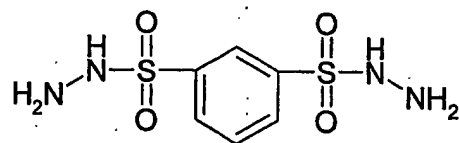
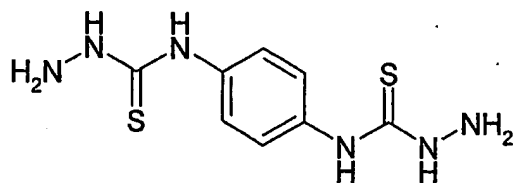
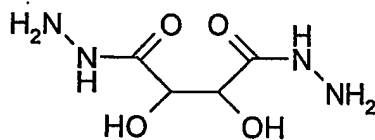
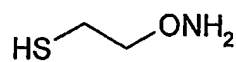
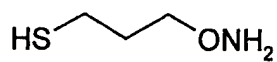
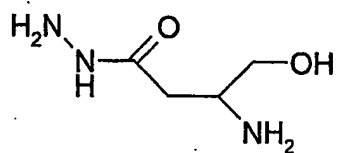
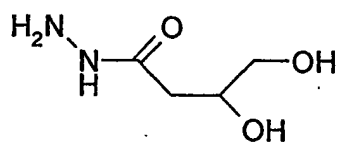
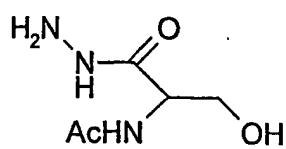
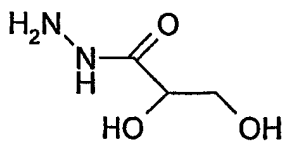
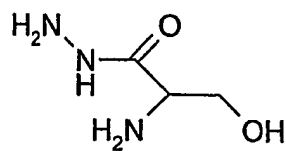
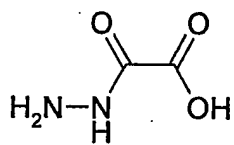
According to another embodiment of the present invention, the NH group bridging R' and R" may be separated from the at least one functional group X by a linear or branched alkyl or cycloalkyl or aryl or or aralkyl or arylcycloalkyl or alkaryl or  
 15 cycloalkylaryl group, wherein these groups may comprise at least one heteroatom such as N, O, S, and wherein these groups may be suitably substituted. The size of the group separating NH, bridging R' and R", and the at least one functional group X may be adapted to the specific needs. Generally, the separating group has generally from 1 to 60, preferably from 1 to 40, more preferably from 1 to 20, more preferably

from 1 to 10, more preferably from 1 to 6 and especially preferably from 1 to 4 carbon atoms. If heteroatoms are present, the separating group comprises generally from 1 to 20, preferably from 1 to 8 and especially preferably from 1 to 4 heteroatoms. According to particularly preferred embodiments of the present invention, the separating group comprises 1 to 4 oxygen atoms. The separating group may comprise an optionally branched alkyl chain or an aryl group or a cycloalkyl group having, e.g., from 5 to 7 carbon atoms, or be an aralkyl group, an alkaryl group where the alkyl part may be a linear and/or cyclic alkyl group. According to an even more preferred embodiment, the separating group is an alkyl chain of from 1 to 20, preferably from 1 to 8, more preferably from 1 to 6, more preferably from 1 to 4 and especially preferably from 2 to 4 carbon atoms. In case heteroatoms are present, a chain comprising 1 to 4 oxygen atoms is particularly preferred.

Specific examples of compounds (II) where X is separated from the NH group bridging R' and R" are, among others,

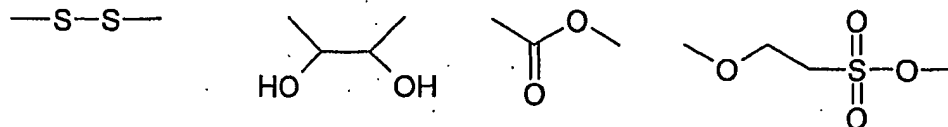






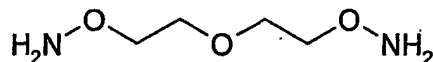
The group separating NH, bridging R' and R'', and the at least one functional group X may be suitably substituted. Preferred substituents are, e.g, halides such as F, Cl, Br or I.

- 5 The group separating NH, bridging R' and R'', and the at least one functional group X may comprise one or more cleavage sites such as

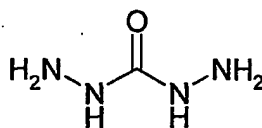


which allow for an easy cleavage of a resulting compound at a pre-determined site.

- According to an especially preferred embodiment of the present invention, the compound (II) is O-[2-(2-aminoxy-ethoxy)-ethyl]-hydroxyl amine
- 10



or carbohydrazide



- Therefore, the present invention also relates to a method as described above wherein compound (II) is O-[2-(2-aminoxy-ethoxy)-ethyl]-hydroxyl amine or carbohy-
- 15 drazide.

In case compound (II) comprises one or more chiral centers, compound (II) may be present in R conformation or in S conformation or as racemic compound with respect to each chiral center.

20

As described above, compound (I) may be reacted with compound (II) as such or with compound (II) which has been reacted with at least one further compound prior to the reaction with compound (I).

The reaction of compound (I) with compound (II) as such may be carried out in at least one suitable solvent. The respective solvent or mixture of two or more solvents may be adapted to the specific needs of the reaction conditions and the chemical nature of compounds (I) and (II). According to an especially preferred embodiment of the present invention, water is used as solvent, either alone or in combination with at least one other solvent. As at least one other solvent, DMSO, DMF, methanol and ethanol may be mentioned. Preferred solvents other than water are DMSO, DMF, methanol and ethanol.

Therefore, the present invention also relates to a method as described above wherein the reaction of compound (I) with compound (II) is carried out in an aqueous system.

The term "aqueous system" as used in the context of the present invention refers to a solvent or a mixture of solvents comprising water in the range of from at least 10 % per weight, preferably at least 50 % per weight, more preferably at least 80 % per weight, even more preferably at least 90 % per weight or up to 100 % per weight, based on the weight of the solvents involved. The preferred reaction medium is water.

As far as the temperatures which are applied during the reaction are concerned, no specific limitations exist given that the reaction results in the desired hydroxyalkyl starch derivative.

In case compound (I) is reacted with compound (II), compound (II) being a hydroxylamine or a hydrazide, the temperature is preferably in the range of from 5 to 45 °C, more preferably in the range of from 10 to 30 °C and especially preferably in the range of from 15 to 25 °C.

In case compound (I) is reacted with compound (II), said reaction being a reductive amination, the temperature is preferably in the range of up to 100 °C, more preferably in the range of from 20 to 95 °C, more preferably in the range of from 25 to 90

°C, more preferably in the range of from 70 to 90 °C and especially preferably in the range of from 75 to 85 °C.

Therefore, the present invention also relates to a method as described above wherein  
5 the reaction of compound (I) and compound (II), compound (II) being a hydroxylamine or a hydrazide, is carried out at a temperature of from 5 to 45 °C.

Therefore, the present invention also relates to a method as described above wherein  
10 the reaction of compound (I) and compound (II), said reaction being a reductive amination, is carried out at a temperature of from 25 to 90 °C.

During the course of the reaction the temperature may be varied, preferably in the above-given ranges, or held essentially constant.

15 The reaction time for the reaction of compound (I) with (II) may be adapted to the specific needs and is generally in the range of from 1 h to 7 d.

In case compound (II) is a hydroxylamine or a hydrazide, the reaction time is preferably in the range of from 1 h to 3 d and more preferably of from 2 h to 48 h.  
20

In case the reaction of compound (I) and compound (II) is a reductive amination, the reaction time is preferably in the range of from 2 h to 7 d.

The pH value for the reaction of compound (I) with (II) may be adapted to the specific needs such as the chemical nature of the reactants.  
25

In case compound (II) is a hydroxylamine or a hydrazide, the pH value is preferably in the range of from 4.5 to 6.5.

30 In case the reaction of compound (I) and compound (II) is a reductive amination, the pH value is preferably in the range of from 8 to 12.

Therefore, the present invention also relates to a method as described above wherein the reaction of compound (I) and compound (II), compound (II) being a hydroxylamine or a hydrazide, is carried out at a pH of from 4.5 to 6.5.

- 5 Therefore, the present invention also relates to a method as described above wherein the reaction of compound (I) and compound (II), said reaction being a reductive amination, is carried out at a pH of from 8 to 12.

- Specific examples of above mentioned reaction conditions are, e.g., a reaction temperature of about 25 °C and a pH of about 5.5 in case compound is a hydroxylamine, and a reaction temperature of about 80 °C and a pH of about 11 in case the reaction of compound (I) and compound (II) is a reductive amination.
- 10

- The suitable pH value of the reaction mixture may be adjusted by adding at least one suitable buffer. Among the preferred buffers, sodium acetate buffer, phosphate or borate buffers may be mentioned.
- 15

- According to a preferred embodiment of the present invention, the reaction product resulting from the reaction of compound (I) with compound (II) is reacted with at least one further compound via the at least one functional group X.
- 20

- If necessary, the at least one functional group X may be protected with at least one suitable protecting group prior to the reaction of compound (I) with compound (II). In this respect, all conceivable protecting groups are possible which prevent the protected compound (II) from reacting with compound (I) via the at least one functional group X. Hence, the protecting group may be chosen depending from the chemical nature of the functional group X to be protected, from, e.g., the solvent the reaction is carried out in or the pH of the reaction mixture. Preferred protecting groups are, among others, the benzyloxycarbonyl group, the tert-butoxycarbonyl group, the methoxyphenyl group, the 2,4-dimethoxyphenyl group, triaryl methyl groups, trityl, the monomethoxytrityl group, the dimethoxytrityl group, the monomethyltrityl group, the dimethyltrityl group, the trifluoroacetyl group, phthalimin compounds, 2-
- 25
- 30

(trialkylsilyl)ethoxy, carbonyl compounds, Fmoc, the tert-butyl group, or trialkyl silyl groups.

5 If two or more different functional groups X are present in compound (II), at least one group may be protected whereas at least one other group may be left unprotected.

10 After the reaction of compound (I) with compound (II), the at least one protecting group may be left in the reaction product or removed by suitable methods such as conventional methods known to the person skilled in the art. If two different functional groups X are protected by suitable protecting groups, it is possible to remove at least one protecting group so as to make at least one functional group X available for further reaction with at least one further compound, and leave at least one other functional group protected until the reaction product of compound (I) with compound  
15 (II) is reacted with the further compound. Afterwards, the protecting group of the functional group still protected may be removed to make the remaining functional group X available for reaction with yet a further compound.

20 The use of at least one protecting group may be important for preventing the reaction from resulting in a hydroxyalkyl starch derivative consisting of a compound (II) which has been reacted with two or more compounds (I), i.e. a multiple HAS substituted compound (II). The same result, however, may be achieved by reacting compound (I) with an excess of compound (II). If an excess amount of compound (II) is used in the process of the present invention, the molar ratio of compound (II) to  
25 compound (I) is preferably in the range of from 2 to 100.

Once the reaction product of the reaction of compound (I) with compound (II) is formed, it may be isolated from the reaction mixture by at least one suitable method. If necessary, the reaction product may be precipitated prior to the isolation by at least  
30 one suitable method.

If the reaction product is precipitated first, it is possible, e.g., to contact the reaction mixture with at least one solvent or solvent mixture other than the solvent or solvent mixture present in the reaction mixture at suitable temperatures. According to a particularly preferred embodiment of the present invention where water is used as solvent, the reaction mixture is contacted with a mixture of ethanol and acetone, preferably a 1:1 mixture, indicating equal volumes of said compounds, at a temperature, preferably in the range of from -20 to +50 °C and especially preferably in the range of from 0 to 25 °C.

- 10 Isolation of the reaction product may be carried out by a suitable process which may comprise one or more steps. According to a preferred embodiment of the present invention, the reaction product is first separated off the reaction mixture or the mixture of the reaction mixture with, e.g., the ethanol-acetone mixture, by a suitable method such as centrifugation or filtration. In a second step, the separated reaction product may be subjected to a further treatment such as an after-treatment like dialysis, centrifugal filtration or pressure filtration, ion exchange chromatography, HPLC, MPLC, gel filtration and/or lyophilisation. According to an even more preferred embodiment, the separated reaction product is first dialysed, preferably against water, and then lyophilized until the solvent content of the reaction product is sufficiently low according to the desired specifications of the product. Lyophilisation may be carried out at temperature of from 20 to 35 °C, preferably of from 25 to 30 °C.

- 25 The thus isolated reaction product of compound (I) and compound (II) may be further reacted with at least one other compound via at least one functional group X comprised in said reaction product.

- 30 Depending on the chemical nature of the functional group X, every conceivable compound capable of forming a chemical linkage with this group X may be used. For this reaction, one or more suitable solvents may be used, and all reaction parameters such as the temperature during the reaction, the reaction time, the ratios of the reactants or the pH value of the reaction mixture may be adapted to the specific needs.

According to a particularly preferred embodiment of the present invention, the at least one compound capable of forming a chemical linkage with the at least one functional group X is a polypeptide or a mixture of at least two different polypeptides.

- 5 Therefore, the present invention also relates to a method as described above wherein the reaction product of compound (I) and compound (II) is reacted with a polypeptide via the functional group X comprised in compound (II).

- 10 According to another particularly preferred embodiment of the present invention, the at least one further compound capable of forming a chemical linkage with the at least one functional group X is a crosslinking compound which is capable of forming a first chemical linkage with the at least one functional group X of the reaction product of compound (I) and compound (II), and a second chemical linkage with second further compound.

15

According to an even more preferred embodiment of the present invention, the second further compound is a polypeptide or a mixture of at least two different polypeptides.

- 20 In the context of this embodiment of the present invention, it is possible to react the reaction product of compound (I) and compound (II), the first hydroxyalkyl starch derivative, with the crosslinking compound to give a second hydroxyalkyl starch derivative. This second hydroxyalkyl starch derivative may be subsequently reacted with the second further compound, preferably a polypeptide, to give a third hydroxyalkyl starch derivative.
- 25

It is, however, also possible to react the reaction product of compound (I) and compound (II), the first hydroxyalkyl starch derivative, with a reaction product of the crosslinking compound with the second further compound, preferably a polypeptide.

30

Therefore, the present invention also relates to a method as described above wherein the reaction product of compounds (I) and (II) is reacted with a further compound,



said further compound being a crosslinking compound, via reaction of a functional group V comprised in the crosslinking compound and a functional group X comprised in the reaction product of compounds (I) and (II).

- 5 Therefore, the present invention also relates to a method as described above wherein the reaction product of compounds (I) and (II) is reacted with a further compound, said further compound being a crosslinking compound, via reaction of a functional group V comprised in the crosslinking compound and a functional group X comprised in the reaction product of compounds (I) and (II), said crosslinking compound  
10 having been reacted with a second further compound prior to the reaction with the reaction product of compounds (I) and (II).

- Therefore, the present invention also relates to a method as described above wherein the second further compound is a polypeptide, preferably erythropoietin, which is  
15 reacted with the crosslinking compound via reaction of a functional group X, comprised in the crosslinking compound.

- Therefore, the present invention also relates to a method as described above wherein compound (II) is reacted with a first further compound, preferably a crosslinking  
20 compound, to give a first reaction product, said first reaction product is reacted with a second further compound to give a second reaction product, and said second reaction product is reacted with compound (I).

- Therefore, the present invention also relates to a method as described above wherein  
25 a first further compound, preferably a crosslinking compound, is reacted with a second further compound, preferably a polypeptide, to give a first reaction product, said first reaction product is reacted with compound (II) to give a second reaction product, and said second reaction product is reacted with compound (I) to give the hydroxyalkyl starch derivative.

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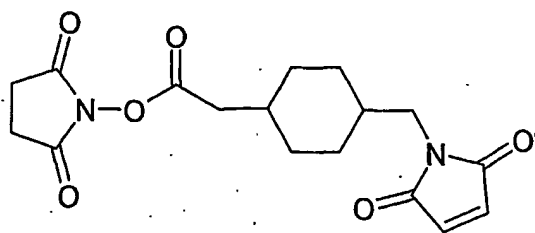
According to especially preferred embodiments of the present invention, the crosslinking compounds are used to form a chemical bridge between compound (II)

or the reaction product of compounds (I) and (II), and a second further compound wherein the functional group of the second further compound which reacts with the crosslinking compound is a -SH group or an aldehyde group or a keto group, and the functional group of compound (II) or the reaction product of compounds (I) and (II) which reacts with the crosslinking compound is a group comprising the structure -NH-, particularly preferably -NH<sub>2</sub>.

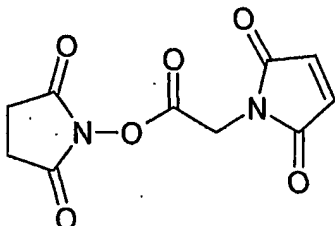
In the context of the present invention, the term "crosslinking compound" relates to chemical compounds which are capable of forming a linkage between compound (II) or the reaction product of compounds (I) and (II), and at least one given second further compound. Depending on the chemical nature of the second further compound, the crosslinking compound comprises at least one functional group V capable of being reacted with the functional group X comprised in compound (II) or the reaction product of compounds (I) and (II), and at least one further functional group which is capable of forming a chemical linkage with the second further compound. This at least one further functional group comprised in the crosslinking compound may be a functional group of the type discussed above with regard to the functional group X.

The crosslinking compound may be used to enlarge the length of the overall chemical bridge between compound (I) and the second further compound, preferably a polypeptide, and/or to influence the chemical nature of the resulting reaction product, either with or without the second further compound, and/or provide the possibility to form a linkage between several second further compounds and the reaction product of compound (I), (II) and the crosslinking compound, and/or to chemically modify the functional group X comprised in the reaction product of compound (I) and (II) so as to render said reaction product capable of reacting with a given further compound.

Thus, embodiments of the present invention which are discussed above and which relate to the chemical modification of the functional group X being a -NH<sub>2</sub> group, with a further compound, e.g.



or

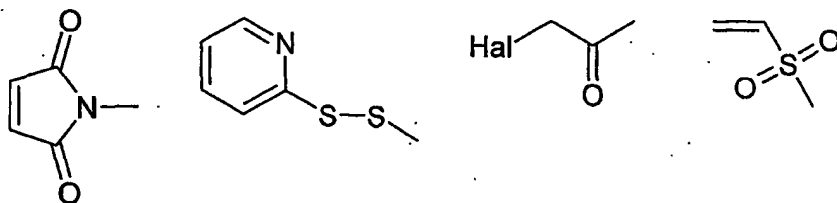


in order to provide the possibility for the reaction with an -SH group comprised in a second further compound, preferably a polypeptide, are specific examples of reacting the reaction product of compounds (I) and (II) with a crosslinking compound.

5

According to a preferred embodiment of the present invention, the functional group V may be a functional group of the type discussed above as group X.

10 According to another preferred embodiment, either functional group X or functional group V is a thio group and functional group V or functional group X is preferably selected from the group consisting of



wherein Hal is Cl, Br, or I, preferably Br or I.

15 According to yet another preferred embodiment, either functional group X or functional group V is selected from the group consisting of an activated ester as described above or a carboxy group which is optionally transformed into an activated ester. In this particular case, the functional group V or the functional group X, respectively, comprises the chemical structure -NH-.

Therefore, the crosslinking compound is a compound having at least two functional groups which are the same or different. In the case of two functional group, the crosslinking compound may be homo-bifunctional or hetero-bifunctional. A homo-bifunctional crosslinking compound, e.g., provides the possibility to form a bridge between the reaction product of compounds (I) with (II) and a second further compound, the reaction product and the further compound having the same type of functional groups. A hetero-bifunctional crosslinking compound, e.g., provides the possibility to form a bridge between the reaction product of compounds (I) with (II) and a second further compound, the reaction product and the further compound having functional groups which are not capable of reacting with each other.

The at least two functional groups of the crosslinking compound may be linked directly together or may be separated by a linear or branched alkyl or cycloalkyl or aryl or aralkyl or arylcycloalkyl or alkaryl or cycloalkylaryl group, wherein these groups may comprise at least one heteroatom such as N, O, S, and wherein these groups may be suitably substituted. The length of the group separating the at least two functional groups of the crosslinking compound may be adapted to the specific needs. Generally, the separating group has from 1 to 60, preferably from 1 to 40, more preferably from 1 to 20, more preferably from 1 to 10, more preferably from 5 to 10 carbon atoms. If heteroatoms are present, the separating group comprises generally from 1 to 20, preferably from 1 to 8 and especially preferably from 1 to 4 heteroatoms. According to an even more preferred embodiment, the separating group is an alkyl or aralkyl chain of from 1 to 20 carbon atoms. Moreover, the crosslinking compound may further comprise at least one cleavage site as discussed above with regard to compound (II).

Other examples of crosslinking compounds which are to be mentioned in the context of the present invention may be categorized, e.g., according to the following list:

Type of crosslinking	Functional group, capa-	Functional group V
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compound	ble of being reacted with a second further com- pound, preferably a polypeptide	
A	Hydrazide (aldehyde- reactive)	Maleimido (SH-reactive)
B	Hydrazide (aldehyde- reactive)	Pyridyldithio (SH- reactive)
C	Iodoalkyl (SH-reactive)	N-succinimide ester (ami- ne-reactive)
D	Bromoalkyl (SH-reactive)	N-succinimide ester (ami- ne-reactive)
E	Maleimido (SH-reactive)	N-succinimide ester (ami- ne-reactive)
F	Pyridyldithio (SH- reactive)	N-succinimide ester (ami- ne-reactive)
G	Vinylsulfone (SH- reactive)	N-succinimide ester (ami- ne-reactive)

In Table 1 at the end of the present description, some preferred examples of crosslinking compounds are listed.

- 5 In case the at least one further compound, e.g. the crosslinking compound, comprises one or more chiral centers, the at least one further compound may be present in R conformation or in S conformation or as racemic compound with respect to each chiral center.
- 10 The term "polypeptide" as used in the context of the present invention refers to a compound which comprises at least 2 amino acids which are linked via a peptide bond, i.e. a bond with structure  $-(C=O)-NH-$ . The polypeptide may be a naturally occurring compound or a polypeptide which does not occur naturally, the latter com-

prising naturally occurring amino acids and/or at least one amino acid which does not naturally occur. The backbone of the polypeptide, the polypeptide chain, may be further substituted with at least one suitable substituent thus having at least one side-chain. The at least one functional group Y may be part of the polypeptide backbone or of at least one substituent of the backbone wherein embodiments are possible comprising at least one functional group being part of the polypeptide backbone and at least one functional group being part of at least one substituent of the polypeptide backbone.

As far as the polypeptide is concerned, there exist no restrictions, given that the polypeptide comprises at least one functional group Y. Said functional group Y may be linked directly to the polypeptide backbone or be part of a side-chain of the backbone. Either side-chain or functional group Y or both may be part of a naturally occurring polypeptide or may be introduced into a naturally occurring polypeptide or into a polypeptide which, at least partially, does not occur naturally, prior to the reaction with the functional group X.

Moreover, the polypeptide can be, at least partly, of any human or animal source. In a preferred embodiment, the polypeptide is of human source.

20

The polypeptide may be a cytokine, especially erythropoietin, an antithrombin (AT) such as AT III, an interleukin, especially interleukin-2, IFN-beta, IFN-alpha, G-CSF, CSF, interleukin-6 and therapeutic antibodies.

According to a preferred embodiment, the polypeptide is an antithrombin (AT), preferably AT III (Levy JH, Weisinger A, Ziomek CA, Echelard Y, Recombinant Antithrombin: Production and Role in Cardiovascular Disorder, Seminars in Thrombosis and Hemostasis 27, 4 (2001) 405-416; Edmunds T, Van Patten SM, Pollock J, Hanson E, Bernasconi R, Higgins E, Manavalan P, Ziomek C, Meade H, McPherson J, Cole ES, Transgenically Produced Human Antithrombin: Structural and Functional Comparison to Human Plasma-Derived Antithrombin, Blood 91, 12 (1998) 4661-4671; Minnema MC, Chang ACK, Jansen PM, Lubbers YTP, Pratt BM, Whittaker

BG, Taylor FB, Hack CE, Friedman B, Recombinant human antithrombin III improves survival and attenuates inflammatory responses in baboons lethally challenged with *Escherichia coli*, Blood 95, 4 (2000) 1117-1123; Van Patten SM, Hanson EH, Bernasconi R, Zhang K, Manavalan P, Cole ES, McPherson JM, Edmunds T, 5 Oxidation of Methionine Residues in Antithrombin, J. Biol. Chemistry 274, 15 (1999) 10268-10276).

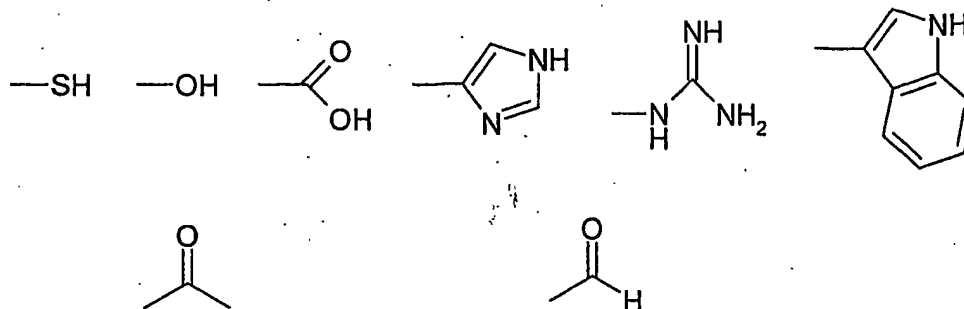
According to another preferred embodiment, the polypeptide is human IFN-beta, in particular IFN-beta 1a (cf. Avonex®, REBIF®) and IFN-beta 1b (cf. 10 BETASERON®).

A further preferred polypeptide is human G-CSF (granulocyte colony stimulating factor). See, e.g., Nagata et al., The chromosomal gene structure and two mRNAs for human granulocyte colony-stimulating factor, EMBO J. 5: 575-581, 1986; Souza et 15 al., Recombinant human granulocyte colony-stimulating factor: effects on normal and leukemic myeloid cells, Science 232 (1986) 61-65; and Herman et al., Characterization, formulation, and stability of Neupogen® (Filgrastim), a recombinant human granulocyte-colony stimulating factor, in: Formulation, characterization, and stability of protein drugs, Rodney Pearlman and Y. John Wang, eds., Plenum Press, 20 New York, 1996, 303-328.

If a mixture of at least two different polypeptides is used, the at least two polypeptides may differ, e.g., in the molecular mass, the number and/or sequence of amino acids, different degrees of glycosylation, the number and/or chemical nature of the 25 substituents or the number of polypeptide chains linked by suitable chemical bonds such as disulfide bridges.

According to a preferred embodiment of the present invention, the reaction product of compound (I) and compound (II), optionally further reacted with a crosslinking 30 compound, is isolated, preferably according to at least one of the above-mentioned processes, and then reacted with a polypeptide having at least one functional group Y capable of being reacted with the at least one functional group X of the reaction

product of compound (I) and compound (II), optionally further reacted with a crosslinking compound, to form at least one chemical linkage. Functional groups Y of polypeptides such as proteins are, e.g.,



or a carbohydrate moiety which may be linked to the polypeptide by N-glycosylation or O-glycosylation.

In the context of the present invention, the term "carbohydrate moiety" refers to hydroxyaldehydes or hydroxyketones as well as to chemical modifications thereof (see Römpp Chemielexikon, Thieme Verlag Stuttgart, Germany, 9<sup>th</sup> edition 1990, Volume 9, pages 2281-2285 and the literature cited therein). Furthermore, it also refers to derivatives of naturally occurring carbohydrate moieties like glucose, galactose, mannose, sialic acid and the like. The term also includes chemically oxidized, naturally occurring carbohydrate moieties. The structure of the oxidized carbohydrate moiety may be cyclic or linear.

The carbohydrate moiety may be linked directly to the polypeptide backbone. Preferably, the carbohydrate moiety is part of a carbohydrate side chain. More preferably, the carbohydrate moiety is the terminal moiety of the carbohydrate side chain.

In an even more preferred embodiment, the carbohydrate moiety is a galactose residue of the carbohydrate side chain, preferably the terminal galactose residue of the carbohydrate side chain. This galactose residue can be made available for reaction with the reaction product of compound (I) and compound (II) by removal of terminal sialic acids, followed by oxidation, as described hereinunder.



In a still further preferred embodiment, the reaction product of compound (I) and (II) is linked to a sialic acid residue of the carbohydrate side chains, preferably the terminal sialic acid residue of the carbohydrate side chain.

- 5    Oxidation of terminal carbohydrate moieties can be performed either chemically or enzymatically.

Methods for the chemical oxidation of carbohydrate moieties of polypeptides are known in the art and include the treatment with periodate (Chamow et al., 1992, J. Biol. Chem., 267, 15916-15922).

By chemically oxidizing, it is in principle possible to oxidize any carbohydrate moiety, being terminally positioned or not. However, by choosing mild conditions (1 mM periodate, 0 °C in contrast to harsh conditions: 10 mM periodate 1h at room temperature), it is possible to preferably oxidize the terminal sialic acid of a carbohydrate side chain.

Alternatively, the carbohydrate moiety may be oxidized enzymatically. Enzymes for the oxidation of the individual carbohydrate moieties are known in the art, e.g. in the case of galactose the enzyme is galactose oxidase. If it is intended to oxidize terminal galactose moieties, it will be eventually necessary to remove terminal sialic acids (partially or completely) if the polypeptide has been produced in cells capable of attaching sialic acids to carbohydrate chains, e.g. in mammalian cells or in cells which have been genetically modified to be capable of attaching sialic acids to carbohydrate chains. Chemical or enzymatic methods for the removal of sialic acids are known in the art (Chaplin and Kennedy (eds.), 1996, Carbohydrate Analysis: a practical approach, especially Chapter 5 Montreuil, Glycoproteins, pages 175-177; IRL Press Practical approach series (ISBN 0-947946-44-3)).

30   Therefore, the present invention also relates to a method as described above wherein the reaction product of compound (I) and compound (II) is reacted with the polypeptide via an oxidized carbohydrate moiety comprised in the polypeptide.

According to another preferred embodiment of the present invention, the functional group of the polypeptide is the thio group. Therefore, the reaction product of compound (I) and (II) may be linked to the polypeptide via a thioether group wherein the S atom can be derived from any thio group comprised in the polypeptide.

The thio group may be present in the polypeptide as such. Moreover, it is possible to introduce a thio group into the polypeptide according to a suitable method. Among others, chemical methods may be mentioned. If a disulfide bridge is present in the polypeptide, it is possible to reduce the -S-S- structure to get a thio group. It is also possible to transform an amino group present in the polypeptide into a SH group by reaction the polypeptide via the amino group with a compound which has at least two different functional groups, one of which is capable of being reacted with the amino group and the other is an SH group or a precursor of an SH group. This modification of an amino group may be regarded as an example where the protein is first reacted with a compound (L) which has at least two different functional groups, one of which is capable of being reacted with the amino group and the other is an SH group, and the resulting reaction product is then reacted with, e.g., a HAS derivative comprising HAS and a compound (D), said derivative comprising a functional group being capable of reacting with the SH group. It is also possible to introduce an SH group by mutation of the polypeptide such as by introducing a cysteine or a suitable SH functional amino acid into the polypeptide or such as removing a cysteine from the polypeptide so as to disable another cysteine in the polypeptide to form a disulfide bridge.

In the context of this embodiment, it is particularly preferred to react the polypeptide with a reaction product which results from the reaction of the reaction product of compounds (I) and (II) with a crosslinking compound.

Therefore, the present invention also relates to a method as described above wherein the reaction product of compound (I) and compound (II) is reacted with a crosslinking compound and the resulting reaction product is further reacted with the poly-

peptide via an oxidized carbohydrate moiety and/or a thio group comprised in the polypeptide.

As an especially preferred polypeptide, erythropoietin (EPO) is used.

5

Therefore, the present invention also relates to a method as described above wherein the polypeptide is erythropoietin.

The EPO can be of any human (see e.g. Inoue, Wada, Takeuchi, 1994, An improved  
10 method for the purification of human erythropoietin with high in vivo activity from the urine of anemic patients, Biol. Pharm. Bull. 17(2), 180-4; Miyake, Kung, Goldwasser, 1977, Purification of human erythropoietin., J. Biol. Chem., 252(15), 5558-64) or another mammalian source and can be obtained by purification from naturally occurring sources like human kidney, embryonic human liver or animal, preferably  
15 monkey kidney. Furthermore, the expression "erythropoietin" or "EPO" encompasses also an EPO variant wherein one or more amino acids (e.g. 1 to 25, preferably 1 to 10, more preferred 1 to 5, most preferred 1 or 2) have been exchanged by another amino acid and which exhibits erythropoietic activity (see e.g. EP 640 619 B1). The measurement of erythropoietic activity is described in the art (for measurement  
20 of activity in vitro see e.g. Fibi et al., 1991, Blood, 77, 1203 ff; Kitamura et al, 1989, J. Cell Phys., 140, 323-334; for measurement of EPO activity in vivo see Ph. Eur. 2001, 911-917; Ph. Eur. 2000, 1316 Erythropoietini solutio concentrata, 780- 785; European Pharmacopoeia (1996/2000); European Pharmacopoeia, 1996, Erythropoietin concentrated solution, Pharmaeuropa., 8, 371-377; Fibi, Hermentin, Pauly, Lauf-  
25 fer, Zettlmeissl., 1995, N- and O-glycosylation muteins of recombinant human erythropoietin secreted from BHK-21 cells, Blood, 85(5), 1229-36; (EPO and modified EPO forms were injected into female NMRI mice (equal amounts of protein 50 ng/mouse) at day 1, 2 and 3 blood samples were taken at day 4 and reticulocytes were determined)). Further publications where tests for the measurement of the activ-  
30 ity of EPO are described Barbone, Aparicio, Anderson, Natarajan, Ritchie, 1994, Reticulocytes measurements as a bioassay for erythropoietin, J. Pharm. Biomed. Anal., 12(4), 515-22; Bowen, Culligan, Beguin, Kendall, Villis, 1994, Estimation of

- effective and total erythropoiesis in myelodysplasia using serum transferrin receptor and erythropoietin concentrations, with automated reticulocyte parameters, *Leukemi*, 8(1), 151-5; Delorme, Lorenzini, Giffin, Martin, Jacobsen, Boone, Elliott, 1992, Role of glycosylation on the secretion and biological activity of erythropoietin, *Biochemistry*, 31(41), 9871-6; Higuchi, Oheda, Kuboniwa, Tomonoh, Shimonaka, Ochi, 1992; Role of sugar chains in the expression of the biological activity of human erythropoietin, *J. Biol. Chem.*, 267(11), 7703-9; Yamaguchi, Akai, Kawanishi, Ueda, Masuda, Sasaki, 1991, effects of site-directed removal of N-glycosylation sites in human erythropoietin on its production and biological properties, *J. Biol. Chem.*, 266(30), 20434-9; Takeuchi, Inoue, Strickland, Kubota, Wada, Shimizu, Hoshi, Kozutsumi, Takasaki, Kobata, 1989, Relationship between sugar chain structure and biological activity of recombinant human erythropoietin produced in Chinese hamster ovary cells, *Proc. Natl. Acad. Sci. USA*, 85(20), 7819-22; Kurtz, Eckardt, 1989, Assay methods for erythropoietin, *Nephron.*, 51(1), 11-4 (German); Zucali, Sulkowski, 1985, Purification of human urinary erythropoietin on controlled-pore glass and silicic acid, *Exp. Hematol.*, 13(3), 833-7; Krystal, 1983, Physical and biological characterization of erythroblast enhancing factor (EEF), a late acting erythropoietic stimulator in serum distinct from erythropoietin, *Exp. Hematol.*, 11(1), 18-31.
- 20 Preferably, the EPO is recombinantly produced. This includes the production in eukaryotic or prokaryotic cells, preferably mammalian, insect, yeast, bacterial cells or in any other cell type which is convenient for the recombinant production of EPO. Furthermore, the EPO may be expressed in transgenic animals (e.g. in body fluids like milk, blood, etc.), in eggs of transgenic birds, especially poultry, preferred
- 25 chicken, or in transgenic plants.

The recombinant production of a polypeptide is known in the art. In general, this includes the transfection of host cells with an appropriate expression vector, the cultivation of the host cells under conditions which enable the production of the polypeptide and the purification of the polypeptide from the host cells. For detailed information see e.g. Krystal, Pankratz, Farber, Smart, 1986, Purification of human erythropoietin to homogeneity by a rapid five-step procedure, *Blood*, 67(1), 71-9;

30

Quelle, Caslake, Burkert, Wojchowski, 1989, High-level expression and purification of a recombinant human erythropoietin produced using a baculovirus vector, *Blood*, 74(2), 652-7; EP 640 619 B1 and EP 668 351 B1.

- 5 In a preferred embodiment, the EPO has the amino acid sequence of human EPO (see EP 148 605 B2).

The EPO may comprise one or more carbohydrate side chains, preferably 1 to 12, more preferably 1 to 9, even more preferably 1 to 6 and particularly 1 to 4, especially  
10 preferably 4 carbohydrate side chains, attached to the EPO via N- and/ or O-linked glycosylation, i.e. the EPO is glycosylated. Usually, when EPO is produced in eukaryotic cells, the polypeptide is posttranslationally glycosylated. Consequently, the carbohydrate side chains may have been attached to the EPO during biosynthesis in mammalian, especially human, insect or yeast cells. The structure and properties of  
15 glycosylated EPO have been extensively studied in the art (see EP 428 267 B1; EP 640 619 B1; Rush, Derby, Smith, Merry, Rogers, Rohde, Katta, 1995, Microheterogeneity of erythropoietin carbohydrate structure, *Anal Chem.*, 67(8), 1442-52; Takeuchi, Kobata, 1991, Structures and functional roles of the sugar chains of human erythropoietins, *Glycobiology*, 1(4), 337-46 (Review).

20

Therefore, the hydroxyalkyl starch derivative according to the present invention may comprise at least one, preferably 1 to 12, more preferably 1 to 9, even more preferably 1 to 6 and particularly preferably 1 to 4 HAS molecules per EPO molecule. The number of HAS-molecules per EPO molecule can be determined by quantitative  
25 carbohydrate compositional analysis using GC-MS after hydrolysis of the product and derivatisation of the resulting monosaccharides (see Chaplin and Kennedy (eds.), 1986, *Carbohydrate Analysis: a practical approach*, IRL Press Practical approach series (ISBN 0-947946-44-3), especially Chapter 1, Monosaccharides, page 1-36; Chapter 2, Oligosaccharides, page 37-53, Chapter 3, Neutral Polysaccharides, page  
30 55-96).

According to an especially preferred embodiment of the present invention, the carbohydrate moiety linked to EPO, is part of a carbohydrate side chain. More preferably, the carbohydrate moiety is the terminal moiety of the carbohydrate side chain. In an even more preferred embodiment, the carbohydrate moiety is a galactose residue  
5 of the carbohydrate side chain, preferably the terminal galactose residue of the carbohydrate side chain. This galactose residue can be made available for reaction with the reaction product of compound (I) and compound (II) by removal of terminal sialic acids, followed by oxidation, as described hereinunder. In a further preferred embodiment, the reaction product of compound (I) and (II) is linked to a sialic acid  
10 residue of the carbohydrate side chains, preferably the terminal sialic acid residue of the carbohydrate side chain. The sialic acid is oxidized as described herein.

Particularly preferably this galactose residue is made available for reaction with the reaction product of compounds (I) and (II) or with the reaction product of the reaction  
15 of the reaction product of compounds (I) and (II) and a crosslinking compound via functional group X by removal of terminal sialic acid followed by oxidation.

As mentioned above, the reaction product of compound (I) and compound (II), optionally reacted with a crosslinking compound, may be reacted with a thio group  
20 comprised in EPO.

It is also possible to react the reaction product of compound (I) and compound (II), optionally reacted with a crosslinking compound, with a thio group as well as with a carbohydrate moiety, each of them comprised in the at least one further compound,  
25 preferably a polypeptide, more preferably erythropoietin.

According to a preferred embodiment, this SH group may be linked to a preferably oxidized carbohydrate moiety, e.g. by using a hydroxylamine derivative, e.g. 2-(aminooxy)ethylmercaptan hydrochloride (Bauer L. et al., 1965, J. Org. Chem., 30,  
30 949) or by using a hydrazide derivative, e.g. thioglycolic acid hydrazide (Whitesides et al., 1977, J. Org. Chem., 42, 332.)

According to a further preferred embodiment, the thio group is preferably introduced in an oxidized carbohydrate moiety of EPO, more preferably an oxidized carbohydrate moiety which is part of a carbohydrate side chain of EPO.

- 5 Preferably, the thio group is derived from a naturally occurring cysteine or from an added cysteine. More preferably, the EPO has the amino acid sequence of human EPO and the naturally occurring cysteines are cysteine 29 and/or 33. In a more preferred embodiment, the reaction product of compound (I) and compound (II), optionally reacted with a crosslinking compound, is reacted with cysteine 29 whereas cysteine 33 is replaced by another amino acid. Alternatively, the reaction product of  
10 compound (I) and compound (II), optionally reacted with a crosslinking compound, is reacted with cysteine 33 whereas cysteine 29 is replaced by another amino acid.

- In the context of the present invention, the term "added cysteines" indicates that the polypeptides, preferably EPO, comprise a cysteine residue which is not present in the  
15 wild-type polypeptide.

In the context of this aspect of the invention, the cysteine may be an additional amino acid added at the N- or C-terminal end of EPO.

20

Furthermore, the added cysteine may have been added by replacing a naturally occurring amino acid by cysteine or a suitably substituted cysteine. Preferably, in the context of this aspect of the invention, the EPO is human EPO and the replaced amino acid residue is serine 126.

25

- The reaction conditions of the reaction of the reaction product of compounds (I) and (II), optionally reacted with a crosslinking compound, with the at least one further compound may be adapted to the specific needs of the respective reaction, such as in the case the at least one further compound is a polypeptide or in the case the at least  
30 one further compound is a crosslinking compound or in the case the at least one further compound is a reaction product of a crosslinking compound and a polypeptide. As buffer compounds, at least one of the above-mentioned compounds may be pref-

erably used. As solvent or mixture of solvents, at least one of the above-mentioned solvents may be preferably used. Isolation and/or after-treatment may be carried out, wherein preferred methods are selected from the methods discussed above.

- 5 If the reaction product of compound (I) and compound (II) is, for example, further reacted with a polypeptide as further compound, preferably EPO, water is preferably used as solvent for the reaction. Additionally to water, at least one further solvent may be present. As preferred possible further solvent, DMSO, DMF, methanol or ethanol may be mentioned.

10

Therefore, the present invention also relates to a method as described above wherein the reaction of the reaction product of compound (I) and compound (II) with a polypeptide, preferably EPO, is carried out in an aqueous system.

- 15 As far as the temperatures which are applied during this reaction are concerned, no specific limitations exist given that the reaction results in the desired hydroxyalkyl starch derivative comprising the reaction product of compounds (I) and (II) reacted with the polypeptide via the at least one functional group X. The temperature of the reaction is preferably in the range of from 4 to 37 °C, more preferably in the range of  
20 from 10 to 30 °C especially preferably in the range of from 15 to 25 °C.

Therefore, the present invention also relates to a method as described above wherein the reaction of the reaction product of compound (I) and compound (II) with the polypeptide is carried out at a temperature of from 4 to 37 °C.

25

During the course of the reaction the temperature may be varied, preferably in the above-given ranges, or held essentially constant.

30

The reaction time for reaction of the reaction product of compound (I) and compound (II) with the polypeptide may be adapted to the specific needs and is generally in the range of from 0.5 to 48 h, preferably in the range of from 2 to 24 h and especially preferably in the range of from 10 to 20 h.



The pH value for the reaction of the reaction product of compound (I) and compound (II) with the polypeptide may be adapted to the specific needs such as the chemical nature of the reactants.

5

If, e.g., the reaction product of compound (I) and (II) is reacted with a further compound via the reaction of a functional group X which is a hydroxylamino group -O-NH<sub>2</sub> with at least one aldehyd group which is comprised in the polypeptide, the pH is preferably in the range of from 4.5 to 6, more preferably at about 5.5.

10

If the reaction product of compound (I) and compound (II) is, for example, further reacted with a crosslinking compound as further compound, preferably EPO, water is preferably used as solvent for the reaction. Additionally to water, at least one further solvent may be present. As preferred possible further solvent, DMSO, DMF, methanol or ethanol may be mentioned.

15

*Therefore, the present invention also relates to a method as described above wherein the reaction of the reaction product of compound (I) and compound (II) with a crosslinking compound is carried out in an aqueous system.*

20

As far as the temperatures which are applied during this reaction are concerned, no specific limitations exist given that the reaction results in the desired hydroxyalkyl starch derivative comprising the reaction product of compounds (I) and (II) reacted with the crosslinking compound via the at least one functional group X. The temperature of the reaction is preferably in the range of from 4 to 37 °C, more preferably in the range of from 10 to 30 °C, especially preferably in the range of from 15 to 25 °C.

25

Therefore, the present invention also relates to a method as described above wherein the reaction of the reaction product of compound (I) and compound (II) with the crosslinking compound is carried out at a temperature of from 4 to 37 °C.

30

During the course of the reaction the temperature may be varied, preferably in the above-given ranges, or held essentially constant.

5 The reaction time for reaction of the reaction product of compound (I) and compound (II) with the crosslinking compound may be adapted to the specific needs and is generally in the range of from 10 min to 10 h, preferably of from 20 min to 5 h and more preferably of from 30 min to 2 h.

10 The pH value for the reaction of the reaction product of compound (I) and compound (II) with the crosslinking compound may be adapted to the specific needs such as the chemical nature of the reactants.

15 If, e.g., the reaction product of compound (I) and (II) is reacted with a crosslinking compound which is a crosslinking compound via the functional group X which is comprised in the reaction product of compound (I) and (II) and is an amino group -NH<sub>2</sub>, the pH is preferably in the range of from 7 to 8.5, more preferably at about 7.2.

20 If the reaction product of the reaction of the reaction product of compounds (I) and (II), and a crosslinking compound is, for example, further reacted with a polypeptide, preferably EPO, water is preferably used as solvent for the reaction. Additionally to water, at least one further solvent may be present. As preferred possible further solvent, DMSO, DMF, methanol or ethanol may be mentioned.

25 Therefore, the present invention also relates to a method as described above wherein the reaction of the reaction product of the compound (I) and compound (II) which is further with reacted with a crosslinking compound, with a polypeptide is carried out in an aqueous system.

30 As far as the temperatures which are applied during this reaction are concerned, no specific limitations exist given that the reaction results in the desired hydroxyalkyl starch derivative comprising the reaction product of compounds (I) and (II), reacted with a crosslinking compound and further reacted with a polypeptide via the at least

one functional group X comprised in the crosslinking compound. The temperature of the reaction is preferably in the range of from 4 to 37 °C, more preferably in the range of from 10 to 30 °C especially preferably in the range of from 15 to 25 °C.

5 Therefore, the present invention also relates to a method as described above wherein the reaction of the reaction product of compound (I) and compound (II) which is further reacted with a crosslinking compound, with the polypeptide is carried out at a temperature of from 4 to 37 °C.

10 During the course of the reaction the temperature may be varied, preferably in the above-given ranges, or held essentially constant.

The reaction time for the reaction of the reaction product of compound (I) and compound (II) which is further reacted with a crosslinking compound, with the polypeptide may be adapted to the specific needs and is generally in the range of from 0.5 to 15 48 h, preferably in the range of from 2 to 24 h and especially preferably in the range of from 10 to 20 h.

The pH value for the reaction of the reaction product of compound (I) and compound 20 (II) which is further reacted with a crosslinking compound, with the polypeptide may be adapted to the specific needs such as the chemical nature of the reactants.

If, e.g., the reaction product of compound (I) and (II) is which is further reacted with a crosslinking compound, is reacted with a polypeptide via the functional group X 25 which is comprised in the crosslinking compound and is an amino group -NH<sub>2</sub>, the pH is preferably in the range of from 7 to 8.5, more preferably at about 7.2.

The suitable pH value of the reaction mixture may be adjusted in each case by adding at least one suitable buffer. Among the preferred buffers, sodium acetate buffer, so- 30 dium phosphate buffer, or borate buffers may be mentioned.

The reaction product resulting from the reaction of the reaction product of compound (I) and compound (II) with the at least one further compound, the at least one further compound being either a polypeptide or a crosslinking compound, and the reaction product further comprising the compounds resulting from the reactions of compound (I), compound (II), a crosslinking compound and a polypeptide, may be isolated from the reaction mixture by at least one suitable method and subjected to at least one further treatment such as at least an after-treatment such as dialysis and/or lyophilization.

Once the above-mentioned reaction product is formed, it may be isolated from the reaction mixture by at least one suitable method.

Isolation of the reaction product may be carried out by a suitable process which may comprise one or more steps.

According to a preferred embodiment of the present invention, where the reaction product does not comprise a polypeptide, the reaction product is first separated off the reaction mixture or the mixture of the reaction mixture by preferably centrifugal filtration. In a second step, the separated reaction product may be subjected to a further treatment such as an after-treatment like dialysis and/or lyophilisation. According to an even more preferred embodiment, the separated reaction product is first dialysed, preferably against water, and then lyophilized until the solvent content of the reaction product is sufficiently low according to the desired specifications of the product.

According to another embodiment of the present invention where the reaction product comprises the polypeptide, the reaction product is preferably isolated as described in Example 7.8.

According to a further embodiment of the present invention, compound (II) is reacted with a further compound prior to the reaction with compound (I), i.e. a derivative of compound (II) is produced by the reaction of compound (II) via the at least one func-

tional group X with at least one further compound comprising at least one functional group Y, as described above, prior to the reaction with compound (I).

If compound (II) is first reacted with a further compound, preferably a polypeptide, more preferably EPO, water is preferably used as solvent for the reaction. Additionally to water, at least one further solvent may be present. As preferred possible further solvent, DMSO, DMF, methanol and ethanol may be mentioned.

Therefore, the present invention also relates to a method as described above wherein the reaction of compound (II), prior to the reaction with compound (I), with a further compound, preferably a polypeptide, even more preferably EPO, is carried out in an aqueous system.

As far as the temperatures which are applied during the reaction are concerned, no specific limitations exist given that the reaction results in the desired derivative of compound (II) comprising the reaction product of compound (II) reacted with at least one further compound via the at least one functional group X, preferably a polypeptide, more preferably EPO. The temperature of the reaction are preferably in the range of from 4 to 37 °C, more preferably in the range of from 10 to 30 °C especially preferably in the range of from 15 to 25 °C.

Therefore, the present invention also relates to a method as described above wherein the reaction compound (II) with the at least one further compound is carried out at a temperature of from 4 to 37 °C.

During the course of the reaction the temperature may be varied, preferably in the above-given ranges, or held essentially constant.

The reaction time, the pH value for reaction of compound (II) with the at least one further compound may be adapted to the specific needs such as the chemical nature of the reactants. The suitable pH value of the reaction mixture may be adjusted by adding at least one suitable buffer. Among the preferred buffers, acetate, phosphate,

or borate buffers such as sodium acetate, sodium phosphate, or sodium borate buffers may be mentioned.

5 The reaction product resulting from the reaction of compound (II) with the at least one further compound may be isolated from the reaction mixture by at least one suitable method and subjected to at least one further treatment such as at least an after-treatment such as dialysis and/or lyophilization.

10 Once the reaction product of reaction compound (II) with the at least one further compound is formed, it may be isolated from the reaction mixture by at least one suitable method.

Isolation of the reaction product may be carried out by a suitable process which may comprise one or more steps as already described above.

15

If desired and/or necessary, the NH group bridging R' and R" of compound (II) may be protected with a suitable protecting group prior to the reaction of compound (II) with the at least one further compound. As protecting group, one of the above-mentioned protecting groups may be used. Prior to the reaction of the reaction product of compound (II) and the at least one further compound such as a polypeptide, preferably EPO, with compound (I), the protecting group is removed by a at least one suitable method.

20

If compound (II) is first reacted with a crosslinking compound or a reaction product of a crosslinking compound and a polypeptide, all reaction conditions may be adjusted to the specific needs of these reactions. Among others, the above-mentioned buffer systems and/or solvents may be used.

25

In a second step, the reaction product of the reaction of compound (II) with the at least one further compound is reacted with compound (I).

30

For this reaction, all reaction conditions may be adjusted to the specific needs of these reactions. Among others, the above-mentioned buffer systems and/or solvents may be used.

- 5 The reaction product resulting from the reaction of the reaction product of compound (II) and the at least one further compound with compound (I) may be isolated from the respective reaction mixture by at least one suitable method and subjected to at least one further treatment such as at least an after-treatment such as dialysis and/or lyophilization. In this context, every suitable method described above may be used.

10

Generally, isolation of the HAS-polypeptide conjugate, either with or without crosslinking compound, can be performed by using known procedures for the purification of natural and recombinant polypeptides such as. size exclusion chromatography, ion-exchange chromatography, RP-HPLC, hydroxyapatite chromatography, hydrophobic interaction chromatography or combinations of at least two methods thereof.

15

The covalent attachment of HAS to the polypeptide can be verified by carbohydrate compositional analysis after hydrolysis of the modified protein.

20

Demonstration of HAS modification at N-linked oligosaccharides of the polypeptide can be accomplished by removal of the HAS modified N-glycans and observation of the predicted shift to higher mobility in SDS-PAGE +/- Western Blotting analysis.

25

HAS modification of the polypeptide at cysteine residues can be demonstrated by the failure to detect the corresponding proteolytic Cys-peptide in RP-HPLC and MALDI/TOF-MS in the proteolytic fragments of the HAS-modified product (Zhou et al., 1998, Application of capillary electrophoresis, liquid chromatography, electrospray-mass spectrometry and matrix-assisted laserdesorption/ionization - time of flight - mass spectrometry to the characterization of recombinant human erythropoietin, Electrophoresis, 19(13), 2348-55). The isolation of the HAS-containing fraction after proteolytic digestion of the Cys-modified polypeptide enables the verification in

30

this fraction of the corresponding peptide by conventional amino acid compositional analysis.

5 All embodiments disclosed above with respect of the HAS-polypeptide of the invention concerning properties of the polypeptide or HAS apply also to the method of the invention for the production of a HAS-polypeptide conjugate. Furthermore, all em-  
bodiments disclosed above with respect to HAS-EPO or the preparation thereof which relate to peptides in general or to HAS apply also to the method of the inven-  
tion for the production of a HAS-polypeptide conjugate.

10

According to an especially preferred embodiment of the present invention hydroxyethyl starch is reacted with a compound (II), preferably selected from the homo- and heterobifunctional compounds described above, and the resulting reaction product is reacted with a glycoprotein, preferably erythropoietin, preferably with the  
15 oxidized terminal carbohydrate moiety of a EPO carbohydrate side chain.

According to another especially preferred embodiment of the present invention hydroxyethyl starch is reacted with a compound (II), preferably selected from the homo- and heterobifunctional compounds described above, to give a first hydroxyethyl starch derivative. This first hydroxyethyl starch derivative is subsequently  
20 reacted with a crosslinking compound to give a second hydroxyethyl starch derivative. This second hydroxyethyl starch derivative is subsequently reacted with a glycoprotein, preferably erythropoietin, preferably with a -SH group comprised in the glycoprotein, to give a third hydroxyethyl starch derivative. Preferably, the crosslink-  
25 ing compound is a heterobifunctional compound. More preferably, the crosslinking compound is reacted with a functional group comprising the structure -NH- which is comprised in the first hydroxyethyl starch derivative. More preferably, this functional group is -NH<sub>2</sub>.

30 One advantage of the present invention is that it is not necessary to use toxicologically critical solvents in at least one reaction step, preferably all reaction steps, the reaction step involved and thus, is not necessary to remove these solvents after the

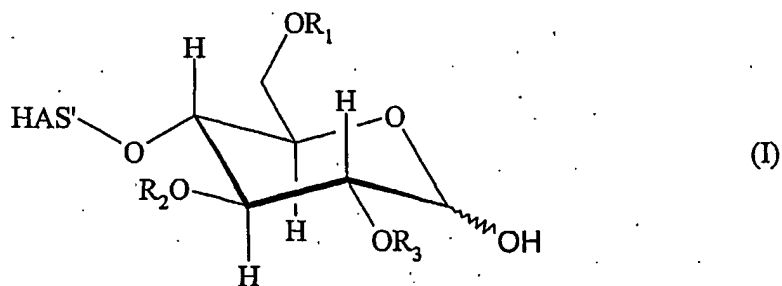


production process in order to avoid the contamination of the products with the solvent. Furthermore, it is not necessary to perform additional quality controls with respect to residual toxicologically critical solvents. If organic solvents, preferably in addition to water, are used, it is preferred to use toxicologically uncritical solvents such as ethanol and/or propylenglycol.

Another advantage of the present invention is that irreversible or reversible structural changes are avoided in the steps where an aqueous system is used as solvent which are otherwise induced by organic solvents. Consequently, polypeptide derivatives obtained according to the method of the invention are different from those prepared in organic solvents such as DMSO.

Furthermore, it has been surprisingly observed that the conjugation of HAS to polypeptides such as EPO in an aqueous solution minimizes or avoids side reactions. Consequently, this embodiment of the method of the invention leads to improved hydroxyalkyl starch products with great purity.

According to another aspect, the present invention also relates to the hydroxy alkyl starch derivative, obtainable by a process comprising reacting hydroxyalkyl starch (HAS) of formula (I)



at its reducing end which is not oxidized prior to said reaction, with a compound of formula (II)



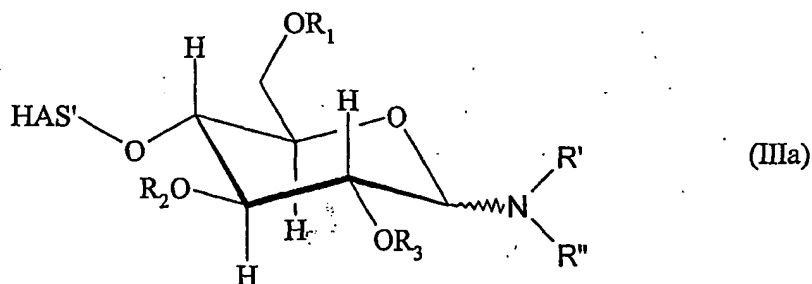
wherein  $\text{R}_1$ ,  $\text{R}_2$  and  $\text{R}_3$  are independently hydrogen or a linear or branched hydroxyalkyl group, and wherein either  $\text{R}'$  or  $\text{R}''$  or  $\text{R}'$  and  $\text{R}''$  comprise at least one

functional group X capable of being reacted with at least one other compound prior to or after the reaction of (I) and (II).

As already described above in the context of the methods of the present invention, O-  
 5 [2-(2-aminoxy-ethoxy)-ethyl]-hydroxyl amine is used as a preferred compound (II),  
 and hydroxyethyl starch is used as a preferred hydroxyalkyl starch.

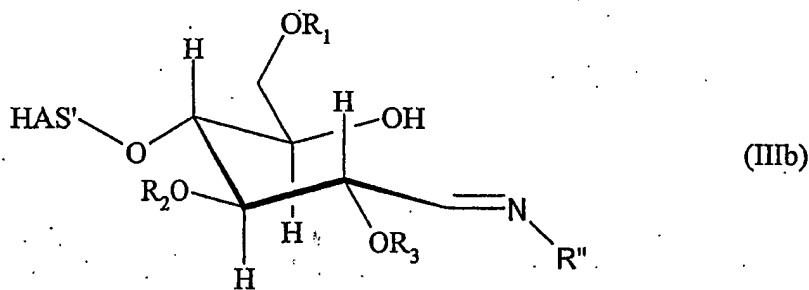
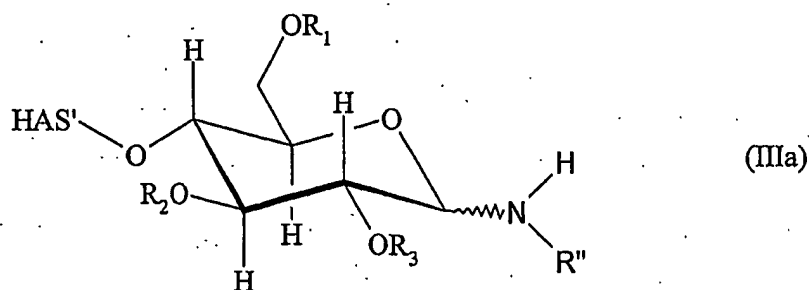
Therefore, the present invention also relates to a hydroxyalkyl starch derivative obtainable by a method wherein hydroxyethyl starch is reacted via its reducing end with  
 10 O-[2-(2-aminoxy-ethoxy)-ethyl]-hydroxyl amine.

Depending on the respective reaction conditions, the solvent or solvent mixture used and/or the residues R' and/or R'' it is possible that the hydroxyalkyl starch derivate obtainable by the method or methods described above may have the following constitutions (IIIa):  
 15



Therefore, the present invention also relates to a hydroxyalkyl starch derivative as described above having a constitution according to formula (IIIa).

20 It is also possible that, e.g. in the case where R' is hydrogen that the hydroxyalkyl starch derivate obtainable by the method or methods described above may have the following constitutions (IIIa) or (IIIb) where (IIIa) and (IIIb) may be both present in the reaction mixture having a certain equilibrium distribution:



Therefore, the present invention also relates to a hydroxyalkyl starch derivative as described above having a constitution according to formula (IIIb).

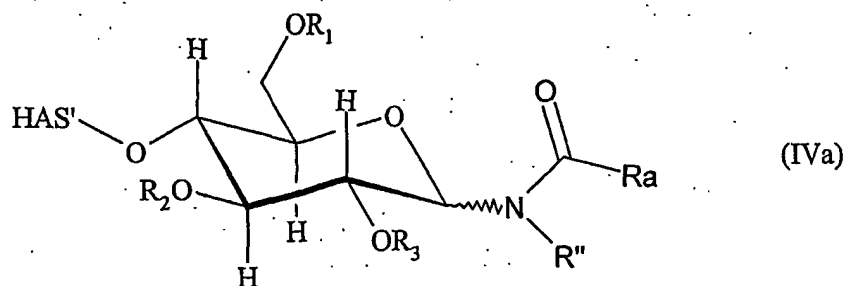
- 5 Moreover, the present invention also relates to a hydroxyalkyl starch derivative as described above being present in a mixture of constitutions according to formulae (IIIa) and (IIIb).

Depending on the reaction conditions and/or the chemical nature of compound (II)  
 10 used for the reaction, the compounds according to formula (IIIa) may be present with the N atom in equatorial or axial position where also a mixture of both forms may be present having a certain equilibrium distribution.

Depending on the reaction conditions and/or the chemical nature of compound (II)  
 15 used for the reaction, the compounds according to formula (IIIb) may be present with the C-N double bond in *E* or *Z* conformation where also a mixture of both forms may be present having a certain equilibrium distribution.

In some cases it may be desirable to stabilize the compound according to formula  
 20 (IIIa). This is especially the case where the compound according to formula (IIIa) is

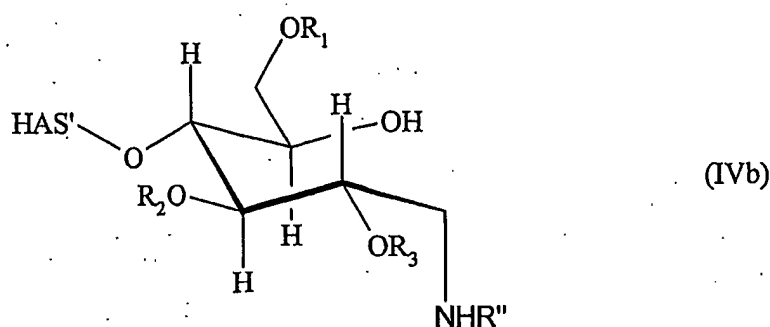
produced and/or used in an aqueous solution. As stabilizing method, acylation of the compound according to formula (IIIa) is particularly preferred, especially in the case where R' is hydrogen. As acylation reagent, all suitable reagents may be used which result in the desired hydroxyalkyl starch derivative according to formula (IVa)



- 5 According to especially preferred embodiments of the present invention, the residue Ra being part of the acylation reagent is methyl. As acylation reagents, carboxylic acid anhydrides, carboxylic acid halides, and carboxylic acid active esters are preferably used.
- 10 Therefore, the present invention also relates to a hydroxyalkyl starch derivative obtainable by a method as described above wherein said derivative has a constitution according to formula (IVa).

The acylation is carried at a temperature in the range of from 0 to 30 °C, preferably  
 15 in the range of from 2 to 20 °C and especially preferably in the range of from 4 to 10 °C.

In other cases it may be desirable to stabilize the compound according to formula (IIIb). This is especially the case where the compound according to formula (IIIb) is  
 20 produced and/or used in an aqueous solution. As stabilizing method, reduction of the compound according to formula (IIIb) is particularly preferred, especially in the case where R' is hydrogen. As reduction reagent, all suitable reagents may be used which result in the desired hydroxyalkyl starch derivative according to formula (IVb)



According to especially preferred embodiments of the present invention, as reduction reagents boro hydrides such as NaCNBH<sub>3</sub> or NaBH<sub>4</sub> are used.

Therefore, the present invention also relates to a hydroxyalkyl starch derivate obtain-  
 5 able by a method as described above wherein said derivative has a constitution ac-  
 cording to formula (IVb).

The reduction is carried at a temperature in the range of from 4 to 100 °C, preferably  
 in the range of from 10 to 90 °C and especially preferably in the range of from 25 to  
 10 80 °C.

The present invention further relates to mixtures of compounds (IIIa) and (IIIb),  
 (IVa) and (IVb), (IIIa) and (IVa), (IIIa) and (IVb), (IIIb) and (IVa), (IIIb) and (IVb),  
 (IIIa) and (IIIb) and (IVa), (IIIa) and (IIIb) and (IVb), (IVa) and (IVb) and (IIIa), and  
 15 (IVa) and (IVb) and (IIIb) wherein (IIIa) and/or (IVa) may be independently present  
 in a conformation where the N atom in equatorial or axial position and/or wherein  
 (IIIb) may be present with the C-N double bond in *E* or *Z* conformation.

According to one aspect of the present invention, compound (I) is reacted with com-  
 20 pound (II) to give a first reaction product. Said first reaction product is then option-  
 ally stabilized according to at least one of the methods described above. The first,  
 optionally stabilized reaction product is then reacted with at least one further com-  
 pound via the reaction of at least one functional group X comprised in R'' of the first  
 reaction product with at least one functional group Y comprised in the at least one

further compound, to give a second reaction product. Said second reaction product is then optionally stabilized according to at least one of the methods described above.

According to yet another aspect of the present invention, the at least one further  
5 compound is a polypeptide or a crosslinking compound or a reaction product of a  
crosslinking compound with a polypeptide. In case the at least one further compound  
is a polypeptide, the functional group Y is comprised in the polypeptide. In case the  
at least one further compound is a crosslinking compound, the functional group Y is  
comprised in the crosslinking compound and optionally also in the polypeptide. In  
10 case the at least one further compound is a reaction product of a crosslinking com-  
pound with a polypeptide, the functional group Y is comprised in the crosslinking  
compound.

According to a further aspect of the present invention, compound (II) is reacted with  
15 at least one further compound via the reaction of at least one functional group X  
comprised in R" of compound (II) with at least one functional group Y comprised in  
the at least one further compound to give a first reaction product. The at least one  
further compound is preferably a polypeptide or a crosslinking compound or a reac-  
tion product of a crosslinking compound with a polypeptide, as discussed above.  
20 Said first reaction product is then reacted with compound (I) via the reaction of the  
reducing end of compound (I) with the NH group of the first reaction product bridg-  
ing the original residues R' and R" of compound (II) to give a second reaction prod-  
uct. Said second reaction product is then optionally stabilized according to at least  
one of the methods described above.

25 According to an especially preferred embodiment of the present invention, hy-  
droxyethyl starch is used as compound (I), O-[2-(2-aminooxy-ethoxy)-ethyl]-  
hydroxyl amine is used as compound (II), and EPO having an oxidized terminal car-  
bohydrate moiety of a carbohydrate side chain is used as further compound. More  
preferably, hydroxyethyl starch is reacted with O-[2-(2-aminooxy-ethoxy)-ethyl]-  
30 hydroxyl amine to give a first hydroxyethyl starch derivate, and that first derivative  
is further reacted with EPO having an oxidized terminal carbohydrate moiety of a

carbohydrate side chain to give a second hydroxyethyl starch derivate. In this specific case, no stabilizing reaction whatsoever has to be carried out.

Therefore, the present invention also relates to a hydroxyalkyl starch derivative obtainable by a method wherein hydroxyethyl starch is reacted via its reducing end with  
5 O-[2-(2-aminoxy-ethoxy)-ethyl]-hydroxyl amine and the reaction product is reacted with erythropietin via the oxidized terminal carbohydrate moiety of a carbohydrate side chain of the erythropietin.

10 According to yet another especially preferred embodiment of the present invention, hydroxyethyl starch is used as compound (I), O-[2-(2-aminoxy-ethoxy)-ethyl]-hydroxyl amine is used as compound (II), a heterobifunctional crosslinking compound having a maleimide group and a N-hydroxy succinimide active ester group, is used, and EPO having at least one -SH group (referred to as ThioEPO) is used as  
15 polypeptide. More preferably, hydroxyethyl starch is reacted with O-[2-(2-aminoxy-ethoxy)-ethyl]-hydroxyl amine to give a first hydroxyethyl starch derivate, that first derivative is further reacted with the N-hydroxy succinimide active ester group of the crosslinking compound to give a second derivative, and that second derivative is reacted via the maleimide group with the ThioEPO to give a third hydroxyethyl starch derivate.  
20

The hydroxyalkyl starch derivative which in the following is referred to as HAS-EPO conjugate and which is formed by reaction of compound (I) with compound (II) and possibly a crosslinking compound and erythropietin, has the advantage that it  
25 exhibits an improved biological stability when compared to the erythropietin before conjugation. This is mainly due to the fact that this hydroxyalkyl starch derivative is less or even not recognized by the removal systems of the liver and kidney and therefore persists in the circulatory system for a longer period of time. Furthermore, since the HAS is attached site-specifically, the risk of destroying the in-vivo biological  
30 activity of EPO by conjugation of HAS to EPO is minimized.

The HAS-EPO conjugate of the invention may exhibit essentially the same in-vitro biological activity as recombinant native EPO, since the in-vitro biological activity only measures binding affinity to the EPO receptor. Methods for determining the in-vitro biological activity are known in the art.

5

Furthermore, the HAS-EPO exhibits a greater in-vivo activity than the EPO used as a starting material for conjugation (unconjugated EPO). Methods for determining the in vivo biological activity are known in the art.

10 The HAS-EPO conjugate may exhibit an in vivo activity of from 110 % to 300 %, preferably from 110 % to 200 %, more preferably from 110 % to 180 % or from 110 to 150 %, most preferably from 110 % to 140 %, if the in-vivo activity of the unconjugated EPO is set as 100 %.

15 Compared to the highly sialylated EPO of Amgen (see EP 428 267 B1), the HAS-EPO exhibits preferably at least 50%, more preferably at least 70 %, even more preferably at least 85 % or at least 95 %, at least 150 %, at least 200 % or at least 300 % of the in vivo activity of the highly sialylated EPO if the in-vivo activity of highly sialylated EPO is set as 100 %. Most preferably, it exhibits at least 95 % of the in  
20 vivo activity of the highly sialylated EPO.

The high in-vivo biological activity of the HAS-EPO conjugate of the invention mainly results from the fact that the HAS-EPO conjugate remains longer in the circulation than the unconjugated EPO because it is less recognized by the removal systems of the liver and because renal clearance is reduced due to the higher molecular weight. Methods for the determination of the in-vivo half life time of EPO in the  
25 circulation are known in the art (Sytkowski, Lunn, Davis, Feldman, Siekman, 1998, Human erythropoietin dimers with markedly enhanced in vivo activity, Proc. Natl. Acad. Sci. USA, 95(3), 1184-8).

30

Consequently, it is a great advantage of the present invention that a HAS-EPO conjugate is provided which may be administered less frequently than the EPO prepara-



tions commercially available at present. While standard EPO preparations have to be administered at least every 3 days, the HAS-EPO conjugate of the invention is preferable administered twice a week, more preferably once a week.

5 Furthermore, the method of the invention has the advantage that an effective EPO derivative can be produced at reduced costs since the method does not comprise extensive and time consuming purification steps resulting in low final yield, e.g. it is not necessary to purify away under-sialylated EPO forms which are known to exhibit low or no in-vivo biological activity.

10

Furthermore, the present invention relates to a pharmaceutical composition comprising, in a therapeutically effective amount, the HAS-polypeptide conjugate, preferably the HAS-EPO conjugate, more preferably the HES-EPO conjugate of the present invention. In a preferred embodiment, the pharmaceutical composition comprises  
15 further at least one pharmaceutically acceptable diluent, adjuvant and/or carrier useful in erythropoietin therapy.

20

Therefore, the present invention also relates to a pharmaceutical composition comprising, in a therapeutically effective amount, a hydroxyalkyl starch derivative as described above wherein the reaction product of compound (I) with compound (II) is reacted via the at least one functional group X comprised in compound (II) with at least one further compound or wherein compound (II) is reacted via the at least one functional group X with at least one further compound prior to the reaction with compound (I) and wherein the at least one further compound is a polypeptide.

25

According to preferred embodiments of the present invention, the polypeptide, preferably erythropoietin is reacted with compound (II) or with the reaction product of compound (I) and compound (II) via a thio group or an oxidized carbohydrate moiety comprised in the polypeptide.

30

According to an even more preferred embodiment of the present invention, the polypeptide, preferably erythropoietin is reacted with compound (II) or with the reaction

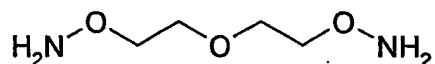
product of compound (I) and compound (II) via an oxidized carbohydrate moiety comprised in the polypeptide.

5 Therefore, the present invention relates to a pharmaceutical composition as described above wherein the polypeptide is reacted with compound (II) or with the reaction product of compound (I) and compound (II) via an oxidized carbohydrate moiety comprised in the polypeptide.

10 According to preferred embodiments, the polypeptide is GCS-F, AT III, IFN-beta or erythropoietin, more preferably erythropoietin.

Therefore, the present invention also relates to a pharmaceutical composition as described above wherein the polypeptide is erythropoietin.

15 According to an especially preferred embodiment of the present invention, the pharmaceutical composition as described above is produced by reacting hydroxyethyl starch in an aqueous medium with a compound according to the following formula



and by reacting the reaction product with erythropoietin.

20 According to a particularly preferred embodiment, the erythropoietin is oxidised with sodium periodate prior to the aforementioned reaction.

25 According to another particularly preferred embodiment, the erythropoietin is partially desialylated and subsequently oxidised with sodium periodate prior to the reaction.

According to a further preferred embodiment of the present invention, pharmaceutical compositions comprising a hydroxyalkyl starch derivative which are produced on the basis of a completely reduced Thio-EPO according to Example 5 are excluded.

30

According to another preferred embodiment, the present invention also relates to a pharmaceutical composition comprising, in a therapeutically effective amount, a hydroxyalkyl starch derivative as described above wherein the reaction product of compound (I) with compound (II) is reacted via the at least one functional group X comprised in compound (II) with at least one further compound or wherein compound (II) is reacted via the at least one functional group X with at least one further compound prior to the reaction with compound (I) and wherein the at least one further compound is a crosslinking compound and the reaction product of the reaction product of compounds (I) and (II) with the crosslinking compound is reacted with a polypeptide.

According to a still further preferred embodiment, the present invention relates to the aforementioned pharmaceutical composition wherein the polypeptide is erythropoietin.

The above-mentioned pharmaceutical composition is especially suitable for the treatment of anemic disorders or hematopoietic dysfunction disorders or diseases related thereto.

A "therapeutically effective amount" as used herein refers to that amount which provides therapeutic effect for a given condition and administration regimen. The administration of erythropoietin isoforms is preferably by parenteral routes. The specific route chosen will depend upon the condition being treated. The administration of erythropoietin isoforms is preferably done as part of a formulation containing a suitable carrier, such as human serum albumin, a suitable diluent, such as a buffered saline solution, and/or a suitable adjuvant. The required dosage will be in amounts sufficient to raise the hematocrit of patients and will vary depending upon the severity of the condition being treated, the method of administration used and the like.

The object of the treatment with the pharmaceutical composition of the invention is preferably an increase of the hemoglobin value of more than 6.8 mmol/l in the blood. For this, the pharmaceutical composition may be administered in a way that the he-

hemoglobin value increases between from 0.6 mmol/l and 1.6 mmol/l per week. If the hemoglobin value exceeds 8.7 mmol/l, the therapy should be preferably interrupted until the hemoglobin value is below 8.1 mmol/l.

- 5 The composition of the invention is preferably used in a formulation suitable for subcutaneous or intravenous or parenteral injection. For this, suitable excipients and carriers are e.g. sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chlorate, polysorbate 80, HSA and water for injection. The composition may be administered three times a week, preferably two times a week, more preferably once  
10 a week, and most preferably every two weeks.

Preferably, the pharmaceutical composition is administered in an amount of 0.01-10 µg/kg body weight of the patient, more preferably 0,1 to 5 µg/kg, 0,1 to 1 µg/kg, or 0.2-0.9 µg/kg, most preferably 0.3-0.7 µg/kg, and most preferred 0.4-0.6 µg/kg body  
15 weight.

In general, preferably between 10 µg and 200 µg, preferably between 15 µg and 100 µg are administered per dosis.

- 20 The invention further relates to a HAS-polypeptide according to the present invention for use in method for treatment of the human or animal body.

The invention further relates to the use of a HAS-EPO conjugate of the present invention for the preparation of a medicament for the treatment of anemic disorders or  
25 hematopoietic dysfunction disorders or diseases related hereto.

The invention is further illustrated by the following examples, tables, and figures which are in no way intended to restrict the scope of the present invention.

### Short description of the Figures

#### Figure 1

- 5 Figure 1 shows an SDS page analysis of the HES-EPO conjugate, produced according to example 4.1.

Lane A: Protein marker Roti®-Mark PRESTAINED (Carl Roth GmbH+Co, Karlsruhe, D); molecular weights (in kD) of the protein marker from top  
10 to bottom: 245, 123, 77, 42, 30, 25.4, and 17.  
Lane B: Crude product after conjugation according to example 4.1.  
Lane C: EPO starting material.

#### Figure 2

- 15 Figure 2 shows an SDS page analysis of the HES-EPO conjugate, produced according to example 4.3.

Lane A: Crude product after conjugation according to example 4.3.  
20 Lane B: EPO starting material.  
Lane C: Protein marker Roti®-Mark PRESTAINED (Carl Roth GmbH+Co, Karlsruhe, D); molecular weights (in kD) of the protein marker from top to bottom: 245, 123, 77, 42, 30, 25.4, and 17.

#### 25 Figure 3

Figure 3 shows an SDS page analysis of HES-EPO conjugates, produced according to examples 6.1 and 6.4.

30 Lane A: Protein marker Roti®-Mark PRESTAINED (Carl Roth GmbH+Co, Karlsruhe, D); molecular weights (in kD) of the protein marker from top to bottom: 245, 123, 77, 42, 30, 25.4, and 17.

Lane B: Crude product after conjugation according to example 6.4.

Lane C: Crude product after conjugation according to example 6.1.

Lane D: EPO starting material.

5 **Figure 4**

Figure 4 shows an SDS page analysis of HES-EPO conjugates, produced according to examples 6.2, 6.3, 6.5, and 6.6.

10 Lane A: Protein marker Roti®-Mark PRESTAINED (Carl Roth GmbH+Co, Karlsruhe, D); molecular weights (in kD) of the protein marker from top to bottom: 245, 123, 77, 42, 30, 25.4, and 17.

Lane B: Crude product after conjugation according to example 6.6, based on Example 1.3 b).

15 Lane C: Crude product after conjugation according to example 6.5, based on Example 1.1 b).

Lane D: Crude product after conjugation according to example 6.6, based on Example 1.3 a).

20 Lane E: Crude product after conjugation according to example 6.5, based on Example 1.1 a).

Lane F: Crude product after conjugation according to example 6.2.

Lane G: Crude product after conjugation according to example 6.3.

Lane K: EPO starting material.

25 **Figure 5**

SDS-PAGE analyses of EPO-GT-1 subjected to mild acid treatment for 5 min. = lane 2; 10 min. = lane 3; 60 min. = lane 4 and untreated EPO = lane 1; the mobility shift of EPO after removal of N-glycans is shown (+PNGASE).

30

**Figure 6**

HPAEC-PAD pattern of oligosaccharides isolated from untreated EPO and from EPO incubated for 5 min., 10 min. and 60 min. under mild acid hydrolysis conditions. Roman numbers I-V indicate the elution position of I = desialylated diantennary structure, II = trisialylated triantennary structures (two isomers), III = tetrasialylated tetraantennary structure + 2 N-acetylglucosamine repeats, IV = tetrasialylated tetraantennary structure + 1 N-acetylglucosamine repeat; V = tetrasialylated tetraantennary structure + without N-acetylglucosamine repeat. The elution area of oligosaccharides structures without, with 1-4 sialic acid is indicated by brackets.

10 **Figure 7**

HPAEC-PAD of N-linked oligosaccharides after desialylation; the elution position of N-acetylneuraminic acid is shown; numbers 1-9 indicate the elution position of standard oligosaccharides: 1 = diantennary; 2 = triantennary (2-4 isomer), 3 = triantennary (2-6 isomer); 4 = tetraantennary; 5 = triantennary plus 1 repeat; 6 = tetraantennary plus 1 repeat; 7 = triantennary plus 2 repeats; 8 = tetraantennary plus 2 repeats and 9 = tetraantennary plus 3 repeats.

**Figure 8**

20

SDS-PAGE analysis of mild treated and untreated EPO which were subjected to periodate oxidation of sialic acid residues. 1 = periodate oxidized without acid treatment; 2 = periodate oxidized 5 min. acid treatment; 3 = periodate oxidized and acid treatment 10 min.; 4 = periodate oxidized without acid treatment; 5 = BRP EPO standard without periodate and without acid treatment.

**Figure 9**

HPAEC-PAD pattern of native oligosaccharides isolated from untreated EPO and from EPO incubated for 5 min and 10 min under mild acid hydrolysis conditions and subsequent periodate treatment. The elution area of oligosaccharides structures without and with 1-4 sialic acid is indicated by brackets 1-5.

**Figure 10**

SDS-PAGE analysis of the time course of HES-modification of **EPO-GT-1-A**: 20  $\mu$ g aliquots of **EPO-GT-1-A** were reacted with hydroxylamine-modified HES derivative **X** for 30 min, 2, 4 and 17 hours. Lane 1 = 30 min reaction time; lane 2 = 2 hour reaction time; lane 3 = 4 hours reaction time; lane 4 = 17 hours reaction time; lane 5 = **EPO-GT-1-A** without HES-modification. Left figure shows the shift in mobility of **EPO-GT-1-A** with increasing incubation time in the presence of the with hydroxylamine-modified HES derivative (flow rate: 1 ml·min<sup>-1</sup>) **X**: Lane 1 = 30 min reaction time; lane 2 = 2 hours reaction time; lane 3 = 4 hours reaction time, lane 4 = 17 hours reaction time; lane 5 = **EPO-GT-1-A** with HES modification. The figure on the right shows analysis of the same samples after their treatment with N-glycosidase.

**Figure 11**

SDS-PAGE analysis of Q-Sepharose fractions of HES-EPO conjugates. Each 1% of the flow-through and 1% of the fraction eluting at high salt concentrations were concentrated in a Speed Vac concentrator and were loaded onto the gels in sample buffer. EPO protein was stained by Coomassie Blue. A = sample I; B = sample II; C = sample III; K = control EPO-GT-1; A1, B1, C1 and K1 indicated the flow-through fraction; A2, B2, C2 and K2 indicates the fraction eluted with high salt concentration.

**Figure 12a**

SDS-PAGE analysis of HES-modified EPO sample A2 (see Fig. 7), control EPO sample K2 and **EPO-GT-1-A** EPO preparation were digested in the presence of N-glycosidase in order to remove N-linked oligosaccharides. All EPO samples showed the mobility shift towards low molecular weight forms lacking or containing O-glycan. A lower ratio of the O-glycosylated and nonglycosylated protein band was



observed for the HES-modified EPO sample A2 after de-*N*-glycosylation and a diffuse protein band was detected around 30 KDa, presumably representing HES-modification at the sialic acid of O-glycan residue (see arrow marked by an asterisk).

5 **Figure 12b**

SDS-PAGE analysis after mild hydrolysis of HES-modified EPO sample A2 (see Fig. 11), control EPO sample K2 and EPO-GT-1A which were untreated or digested in the presence of N-glycosidase in order to remove N-linked oligosaccharides (see 10 **Figure 12a**). Both high molecular weight form of A2 before and A after N.glycosidase treatment (see brackets with and without arrow) disappeared upon acid treatment of the samples. The BRP EPO standard which was run for comparison was not subjected to mild acid treatment.

15 **Figure 13**

HPAEC-PAD analysis of N-linked oligosaccharide material liberated from HES-modified sample A, from EPO-GT-1-A and from a control EPO sample incubated with unmodified HES (K). Roman numbers I-V indicate the elution position of I = 20 disialylated diantennary structure, II = trisialylated triantennary structures (two isomers), III = tetrasialylated tetraantennary structure + 2 N-acetylactosamine repeats, IV = tetrasialylated tetraantennary structure + 1 N-acetylactosamine repeat, V = tetrasialylated tetraantennary structure + without N-acetylactosamine repeat; brackets indicate the elution area of di-, tri- and tetrasialylated N-glycans as reported in the 25 legends of Figs. 6 and 9.

**Figure 14**

HPAEC-PAD analysis of N-linked oligosaccharide material liberated from HES-modified sample A, from EPO-GT-1A and from a control EPO sample (K) incubated 30 with unmodified HES. The retention times of a mixture of standard oligosaccharides is shown: numbers 1-9 indicate the elution position of standard oligosaccharides: 1 =

diantennary; 2 = triantennary (2-4 isomer); 3 = triantennary (2-6 isomer); 4 = tetraantennary; 5 = triantennary plus 1 repeat; 6 = tetraantennary plus 1 repeat; 7 = triantennary plus 2 repeats; 8 = tetraantennary plus 2 repeats and 9 = tetraantennary plus 3 repeats.

5

#### Figures 15 to 21

Figures 15 to 21 represent MALDI/TOF mass spectra of the enzymatically liberated and chemically desialylated N-glycans isolated from HES-modified EPO and control  
10 EPO preparations. Major signals at  $m/z$  1809.7, 2174.8, 2539.9, 2905.0 and 3270.1 ( $[M+Na]^+$ ) correspond to di- to tetraantennary complex-type N-glycan structures with no, one or two N-acetylglucosamine repeats accompanied by weak signals due to loss of fucose or galactose which are due to acid hydrolysis conditions employed for the desialylation of samples for MS analysis.

15

#### Figure 15

MALDI/TOF spectrum: desialylated oligosaccharides of HES-modified EPO A2.

20 **Figure 16**

MALDI/TOF spectrum: desialylated oligosaccharides of EPO GT-1-A.

#### Figure 17

25

MALDI/TOF spectrum: desialylated oligosaccharides of EPO K2.

#### Figure 18

30 **MALDI/TOF spectrum: desialylated oligosaccharides of EPO-GT-1.**

#### Figure 19

MALDI/TOF spectrum: desialylated oligosaccharides of EPO-GT-1 subjected to acid hydrolysis for 5 min.

**5 Figure 20**

MALDI/TOF spectrum: desialylated oligosaccharides of EPO-GT-1 subjected to acid hydrolysis for 10 min.

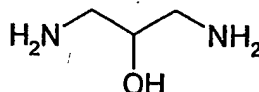
**10 Figure 21**

MALDI/TOF spectrum: desialylated oligosaccharides of EPO-GT-1 subjected to acid hydrolysis for 60 min.

## Examples

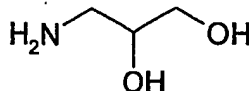
### Example 1: Formation of hydroxyethyl starch derivatives by reductive amination

#### Example 1.1 Reaction of hydroxyethyl starch with 1,3-diamino-2-hydroxy propane



- 10 a) To a solution of 200 mg hydroxyethyl starch (HES18/0.4 (MW = 18,000 D, DS=0.4)) in 5 ml water, 0.83 mmol 1,3-diamino-2-hydroxy propane and 50 mg sodium cyanoborohydrate NaCNBH<sub>3</sub> were added. The resulting mixture was incubated at 80 °C for 17 h. The reaction mixture was added to 160 ml of a cold 1:1 mixture of acetone and ethanol (v/v). The precipitate was collected by
- 15 centrifugation and dialysed for 4 d against water (SnakeSkin dialysis tubing, 3.5 KD cut off, Perbio Science Deutschland GmbH, Bonn, D), and lyophilized.
- b) Incubation of the mixture resulting from adding 0.83 mmol 1,3-diamino-2-hydroxy propane and 50 mg sodium cyanoborohydrate NaCNBH<sub>3</sub> to the solution of 200 mg hydroxyethyl starch was also possible and carried out at 25 °C
- 20 for 3 d.

#### Example 1.2 Reaction of hydroxyethyl starch with 1,2-dihydroxy-3-amino propane



25

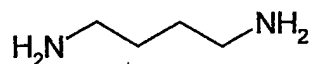
- a) To a solution of 200 mg hydroxyethyl starch (HES18/0.4 (MW = 18,000 D, DS=0.4)) in 5 ml water, 0.83 mmol 1,2-dihydroxy-3-amino propane and 50 mg

sodium cyanoborohydrate  $\text{NaCNBH}_3$  were added. The resulting mixture was incubated at 80 °C for 17 h. The reaction mixture was added to 160 ml of a cold 1:1 mixture of acetone and ethanol (v/v). The precipitate was collected by centrifugation and dialysed for 4 d against water (SnakeSkin dialysis tubing, 3.5 KD cut off, Perbio Science Deutschland GmbH, Bonn, D), and lyophilized.

- b) Incubation of the mixture resulting from adding 0.83 mmol 1,2-dihydroxy-3-amino propane and 50 mg sodium cyanoborohydrate  $\text{NaCNBH}_3$  to the solution of 200 mg hydroxyethyl starch was also possible and carried out at 25 °C for 3 d.

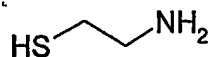
The reaction of 1,2-dihydroxy-3-amino propane with HES was confirmed indirectly by quantification of formaldehyde, resulting from the oxidative cleavage of the 1,2-diole in the reaction product by periodate as described by G. Avigad, Anal. Biochem. 134 (1983) 449-504.

**Example 1.3 Reaction of hydroxyethyl starch with 1,4-diamino butane**



- a) To a solution of 200 mg hydroxyethyl starch (HES18/0.4 (MW = 18,000 D, DS=0.4)) in 5 ml water, 0.83 mmol 1,4-diamino butane and 50 mg sodium cyanoborohydrate  $\text{NaCNBH}_3$  were added. The resulting mixture was incubated at 80 °C for 17 h. The reaction mixture was added to 160 ml of a cold 1:1 mixture of acetone and ethanol (v/v). The precipitate was collected by centrifugation and dialysed for 4 d against water (SnakeSkin dialysis tubing, 3.5 KD cut off, Perbio Science Deutschland GmbH, Bonn, D), and lyophilized.
- b) Incubation of the mixture resulting from adding 0.83 mmol 1,4-diamino butane and 50 mg sodium cyanoborohydrate  $\text{NaCNBH}_3$  to the solution of 200 mg hydroxyethyl starch was also possible and carried out at 25 °C for 3 d.

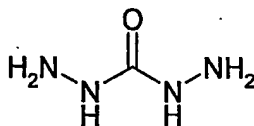
**Example 1.4    Reaction of hydroxyethyl starch with 1-mercapto-2-amino ethane**



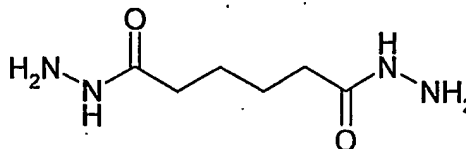
- 5        a)    To a solution of 200 mg hydroxyethyl starch (HES18/0.4 (MW = 18,000 D, DS=0.4)) in 5 ml water, 0.83 mmol 1-mercapto-2-amino ethane and 50 mg sodium cyanoborohydrate NaCNBH<sub>3</sub> were added. The resulting mixture was incubated at 80 °C for 17 h. The reaction mixture was added to 160 ml of a cold 1:1 mixture of acetone and ethanol (v/v). The precipitate was collected by centrifugation and dialysed for 4 d against water (SnakeSkin dialysis tubing, 3.5
- 10        KD cut off, Perbio Science Deutschland GmbH, Bonn, D), and lyophilized.
- 15        b)    Incubation of the mixture resulting from adding 0.83 mmol 1-mercapto-2-amino ethane and 50 mg sodium cyanoborohydrate NaCNBH<sub>3</sub> to the solution of 200 mg hydroxyethyl starch was also possible and carried out at 25 °C for 3 d.

**Example 2:    Formation of hydroxyethyl starch derivatives by conjugation**

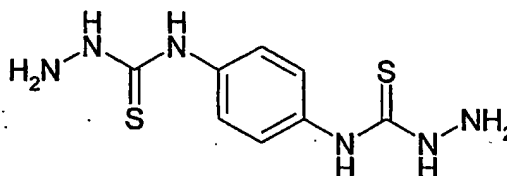
**Example 2.1:    Reaction of hydroxyethyl starch with carbohydrazide**



- 20        0.96 g of HES18/0.4 (MW = 18,000 D, DS=0.4) were dissolved in 8 ml aqueous 0.1 M sodium acetate buffer, pH 5.2, and 8 mmol carbohydrazide (Sigma Aldrich, Taufkirchen, D) were added. After stirring for 18 h at 25 °C, the reaction mixture was added to 160 ml of a cold 1:1 mixture of acetone and ethanol (v/v). The precipitated product was collected by centrifugation, re-dissolved in 40 ml water, and dialysed for 3 d against water (SnakeSkin dialysis tubing, 3.5 KD cut off, Perbio Science
- 25        Deutschland GmbH, Bonn, D), and lyophilized.

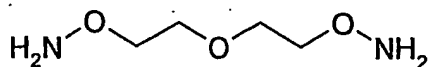
**Example 2.2: Reaction of hydroxyethyl starch with adepic dihydrazide**

0.96 g of HES18/0.4 (MW = 18,000 D, DS=0.4) were dissolved in 8 ml aqueous 0.1 M sodium acetate buffer, pH 5.2, and 8 mmol adepic dihydrazide (Lancaster Synthesis, Frankfurt/Main, D) were added. After stirring for 18 h at 25 °C, the reaction mixture was added to 160 ml of a cold 1:1 mixture of acetone and ethanol (v/v). The precipitated product was collected by centrifugation, re-dissolved in 40 ml water, and dialysed for 3 d against water (SnakeSkin dialysis tubing, 3.5 KD cut off, Perbio Science Deutschland GmbH, Bonn, D), and lyophilized.

**Example 2.3: Reaction of hydroxyethyl starch with 1,4-phenylene-bis-3-thiosemicarbazide**

0.96 g of HES18/0.4 (MW = 18,000 D, DS=0.4) were dissolved in 8 ml aqueous 0.1 M sodium acetate buffer, pH 5.2, and 8 mmol 1,4-phenylene-bis-3-thiosemicarbazide (Lancaster Synthesis, Frankfurt/Main, D) were added. After stirring for 18 h at 25 °C, 8 ml water was added to the reaction mixture, and the suspension was centrifuged for 15 min at 4,500 rpm. The clear supernatant was decanted and subsequently added to 160 ml of a cold 1:1 mixture of acetone and ethanol (v/v). The precipitated product was collected by centrifugation, re-dissolved in 40 ml water, and centrifuged for 15 min at 4,500 rpm. The clear supernatant was dialysed for 3 d against water (SnakeSkin dialysis tubing, 3.5 KD cut off, Perbio Science Deutschland GmbH, Bonn, D), and lyophilized.

**Example 2.4: Reaction of hydroxyethyl starch with O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine**



O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine was synthesized as described in  
5 Boturyn et al. Tetrahedron 53 (1997) p. 5485-5492 in 2 steps from commercially available materials.

0.96 g of HES18/0.4 (MW = 18,000 D, DS=0.4) were dissolved in 8 ml aqueous 0.1  
M sodium acetate buffer, pH 5.2, and 8 mmol O-[2-(2-aminooxy-ethoxy)-ethyl]-  
10 hydroxyl amine were added. After stirring for 18 h at 25 °C, the reaction mixture was added to 160 ml of a cold 1:1 mixture of acetone and ethanol (v/v). The precipitated product was collected by centrifugation, re-dissolved in 40 ml water, and dialysed for 3 d against water (SnakeSkin dialysis tubing, 3.5 KD cut off, Perbio Science Deutschland GmbH, Bonn, D), and lyophilized.

15

**Example 3 Oxidation of erythropoietin**

Oxidized erythropoietin was produced as described in Example 7. As oxidised  
20 erythropoietin, EPO-GT-1-A as described in Example 7.11(c) was used (EPO-GT-1 without acid hydrolysis, treated with mild periodate oxidation).

**Example 4: Conjugation of hydroxyethyl starch derivatives with oxidized erythropoietin of example 3**

25

**Example 4.1 Reaction of oxidized erythropoietin with the reaction product of example 2.1**



Oxidized EPO (1.055 µg/µl) in 20 mM PBS buffer was adjusted to pH 5.3 with 5 M sodium acetate buffer, pH 5.2. To 19 µl of the EPO solution, 18 µl of a solution of the HES derivate as produced according to example 2.1 (MW 18 kD; 18.7 µg/µl in 0.1 M sodium acetate buffer, pH 5.2) was added, and the mixture was incubated for 16 h at 25 °C. After lyophilisation, the crude product was analyzed by SDS-Page with NuPAGE 10% Bis-Tris Gels/MOPS buffer (Invitrogen, Carlsbad, CA, USA) as described in the instructions given by Invitrogen. The gel is stained with Roti-Blue Coomassie staining reagent (Roth, Karlsruhe, D) overnight.

The experimental result is shown in Fig. 1. A successful conjugation is indicated by the migration of the protein band to higher molecular weights. The increased bandwidth is due to the molecular weight distribution of the HES derivatives used and the number of HES derivatives linked to the protein.

**Example 4.2      Reaction of oxidized erythropoietin with the reaction product of example 2.3**

Oxidized EPO (1.055 µg/µl) in 20 mM PBS buffer was adjusted to pH 5.3 with 5 M sodium acetate buffer, pH 5.2. To 19 µl of the EPO solution, 18 µl of a solution of the HES derivate as produced according to example 2.3 (MW 18 kD; 18.7 µg/µl in 0.1 M sodium acetate buffer, pH 5.2) was added, and the mixture was incubated for 16 h at 25 °C. After lyophilisation, the crude product was analyzed by SDS-Page with NuPAGE 10% Bis-Tris Gels/MOPS buffer (Invitrogen, Carlsbad, CA, USA) as described in the instructions given by Invitrogen.

**Example 4.3      Reaction of oxidized erythropoietin with the reaction product of example 2.4**

Oxidized EPO (1.055 µg/µl) in 20 mM PBS buffer was adjusted to pH 5.3 with 5 M sodium acetate buffer, pH 5.2. To 19 µl of the EPO solution, 18 µl of a solution of the HES derivate as produced according to example 2.4 (MW 18 kD; 18.7 µg/µl in 0.1 M sodium acetate buffer, pH 5.2) was added, and the mixture was incubated for

16 h at 25 °C. After lyophilisation, the crude product was analyzed by SDS-Page with NuPAGE 10% Bis-Tris Gels/MOPS buffer (Invitrogen, Carlsbad, CA, USA) as described in the instructions given by Invitrogen. The gel is stained with Roti-Blue Coomassie staining reagent (Roth, Karlsruhe, D) overnight.

5

The experimental result is shown in Fig. 2. A successful conjugation is indicated by the migration of the protein band to higher molecular weights. The increased bandwidth is due to the molecular weight distribution of the HES derivatives used and the number of HES derivatives linked to the protein.

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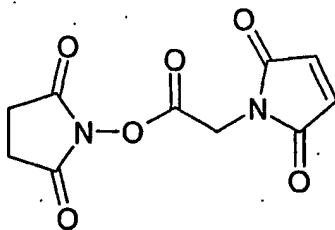
#### **Example 5      Formation of Thio-EPO by reduction of erythropoietin**

241.5 µg erythropoietin (EPO-GT-1, see Example 7) in 500 µl of a 0.1 M sodium borate buffer, 5 mM EDTA, 10 mM DTT (Lancaster, Morcambe, UK), pH 8.3, were incubated for 1 h at 37 °C. The DTT was removed by centrifugal filtration with a VIVASPIN 0.5 ml concentrator, 10 KD MWCO (VIVASCIENCE, Hannover, D) at 13,000 rpm, subsequent washing 3 times with the borate buffer and twice with a phosphate buffer (0.1 M, 9.15 M NaCl, 50 mM EDTA, pH 7.2).

20

#### **Example 6:      Conjugation of hydroxyethyl starch derivatives with thio-erythropoietin using a crosslinking compound**

25 In each of the following examples, N-(alpha-maleimidoacetoxy) succinimide ester (AMAS)



was used as crosslinking compound.

**Example 6.1     Reaction of oxidized erythropoietin with the reaction product of example 2.1 and the crosslinking compound**

- 5     To 50 nmol HES derivate as produced according to example 2.1 and dissolved in 200 µl of a 0.1 M sodium phosphate buffer (0.1 M, 9.15 M NaCl, 50 mM EDTA, pH 7.2), 10 µl of a solution of 2.5 µmol AMAS (Sigma Aldrich, Taufkirchen, D) in DMSO were added. The clear solution was incubated for 80 min at 25 °C and 20 min at 40 °C. Remaining AMAS was removed by centrifugal filtration with a VIVASPIN
- 10    0.5 ml concentrator, 5 KD MWCO (VIVASCIENCE, Hannover, D) at 13,000 rpm, washing 4 times and 30 min each with the phosphate buffer.

To the residual solution, 15 µg of ThioEPO as produced according to example 5 (1 µg/µl in phosphate buffer) were added, and the mixture was incubated for 16 h at 25

15    °C. After lyophilisation, the crude product was analysed by SDS-Page with NuPAGE 10% Bis-Tris Gels/MOPS buffer (Invitrogen, Carlsbad, USA) as described in the instructions given by Invitrogen. The gel is stained with Roti-Blue Coomassie staining reagent (Roth, Karlsruhe, D) overnight.

- 20    The experimental result is shown in Fig. 3. A successful conjugation is indicated by the migration of the protein band to higher molecular weights. The increased bandwidth is due to the molecular weight distribution of the HES derivatives used and the number of HES derivatives linked to the protein.

25    **Example 6.2     Reaction of oxidized erythropoietin with the reaction product of example 2.2 and the crosslinking compound**

- To 50 nmol HES derivate as produced according to example 2.2 and dissolved in 200 µl of a 0.1 M sodium phosphate buffer (0.1 M, 9.15 M NaCl, 50 mM EDTA, pH
- 30    7.2), 10 µl of a solution of 2.5 µmol AMAS (Sigma Aldrich, Taufkirchen, D) in DMSO were added. The clear solution was incubated for 80 min at 25 °C and 20 min at 40 °C. Remaining AMAS was removed by centrifugal filtration with a VIVASPIN

0.5 ml concentrator, 5 KD MWCO (VIVASCIENCE, Hannover, D) at 13,000 rpm, washing 4 times and 30 min each with the phosphate buffer.

To the residual solution, 15 µg of ThioEPO as produced according to example 5 (1 µg/µl in phosphate buffer) were added, and the mixture was incubated for 16 h at 25 °C. After lyophilisation, the crude product was analysed by SDS-Page with NuPAGE 10% Bis-Tris Gels/MOPS buffer (Invitrogen, Carlsbad, USA) as described in the instructions given by Invitrogen. The gel is stained with Roti-Blue Coomassie staining reagent (Roth, Karlsruhe, D) overnight.

10

The experimental result is shown in Fig 4. A successful conjugation is indicated by the migration of the protein band to higher molecular weights. The increased bandwidth is due to the molecular weight distribution of the HES derivatives used and the number of HES derivatives linked to the protein.

15

**Example 6.3      Reaction of oxidized erythropoietin with the reaction product of example 2.3 and the crosslinking compound**

To 50 nmol HES derivate as produced according to example 2.3 and dissolved in 200 µl of a 0.1 M sodium phosphate buffer (0.1 M, 9.15 M NaCl, 50 mM EDTA, pH 7.2), 10 µl of a solution of 2.5 µmol AMAS (Sigma Aldrich, Taufkirchen, D) in DMSO were added. The clear solution was incubated for 80 min at 25 °C and 20 min at 40 °C. Remaining AMAS was removed by centrifugal filtration with a VIVASPIN 0.5 ml concentrator, 5 KD MWCO (VIVASCIENCE, Hannover, D) at 13,000 rpm, washing 4 times and 30 min each with the phosphate buffer.

25

To the residual solution, 15 µg of ThioEPO as produced according to example 5 (1 µg/µl in phosphate buffer) were added, and the mixture was incubated for 16 h at 25 °C. After lyophilisation, the crude product was analysed by SDS-Page with NuPAGE 10% Bis-Tris Gels/MOPS buffer (Invitrogen, Carlsbad, USA) as described in the instructions given by Invitrogen. The gel is stained with Roti-Blue Coomassie staining reagent (Roth, Karlsruhe, D) overnight.

30

The experimental result is shown in Fig. 4. A successful conjugation is indicated by the migration of the protein band to higher molecular weights. The increased band-width is due to the molecular weight distribution of the HES derivatives used and the number of HES derivatives linked to the protein.

**Example 6.4      Reaction of oxidized erythropoietin with the reaction product of example 2.4 and the crosslinking compound**

To 50 nmol HES derivate as produced according to example 2.4 and dissolved in 200 µl of a 0.1 M sodium phosphate buffer (0.1 M, 9.15 M NaCl, 50 mM EDTA, pH 7.2), 10 µl of a solution of 2.5 µmol AMAS (Sigma Aldrich, Taufkirchen, D) in DMSO were added. The clear solution was incubated for 80 min at 25 °C and 20 min at 40 °C. Remaining AMAS was removed by centrifugal filtration with a VIVASPIN 0.5 ml concentrator, 5 KD MWCO (VIVASCIENCE, Hannover, D) at 13,000 rpm, washing 4 times and 30 min each with the phosphate buffer.

To the residual solution, 15 µg of ThioEPO as produced according to example 5 (1 µg/µl in phosphate buffer) were added, and the mixture was incubated for 16 h at 25 °C. After lyophilisation, the crude product was analysed by SDS-Page with NuPAGE 10% Bis-Tris Gels/MOPS buffer (Invitrogen, Carlsbad, USA) as described in the instructions given by Invitrogen. The gel is stained with Roti-Blue Coomassie staining reagent (Roth, Karlsruhe, D) overnight.

The experimental result is shown in Fig 3. A successful conjugation is indicated by the migration of the protein band to higher molecular weights. The increased band-width is due to the molecular weight distribution of the HES derivatives used and the number of HES derivatives linked to the protein.

**Example 6.5      Reaction of oxidized erythropoietin with the reaction product of example 1.1 and the crosslinking compound**

To 50 nmol HES derivate as produced according to example 1.1, at incubation conditions of 80 °C and 17 h (Example 1.1 a)) as well as of 25 °C and 3 d (Example 1.1 b)), and dissolved in 200 µl of a 0.1 M sodium phosphate buffer (0.1 M, 9.15 M NaCl, 50 mM EDTA, pH 7.2), 10 µl of a solution of 2.5 µmol AMAS (Sigma Aldrich, Taufkirchen, D) in DMSO were added. The clear solution was incubated for 80 min at 25 °C and 20 min at 40 °C. Remaining AMAS was removed by centrifugal filtration with a VIVASPIN 0.5 ml concentrator, 5 KD MWCO (VIVASCIENCE, Hannover, D) at 13,000 rpm, washing 4 times and 30 min each with the phosphate buffer.

To the residual solution, 15 µg of ThioEPO as produced according to example 5 (1 µg/µl in phosphate buffer) were added, and the mixture was incubated for 16 h at 25 °C. After lyophilisation, the crude product was analysed by SDS-Page with NuPAGE 10% Bis-Tris Gels/MOPS buffer (Invitrogen, Carlsbad, USA) as described in the instructions given by Invitrogen. The gel is stained with Roti-Blue Coomassie staining reagent (Roth, Karlsruhe, D) overnight.

The experimental result is shown in Fig. 4. A successful conjugation is indicated by the migration of the protein band to higher molecular weights. The increased bandwidth is due to the molecular weight distribution of the HES derivatives used and the number of HES derivatives linked to the protein.

**Example 6.6      Reaction of oxidized erythropoietin with the reaction product of example 1.3 and the crosslinking compound**

To 50 nmol HES derivate as produced according to example 1.3, at incubation conditions of 80 °C and 17 h (Example 1.3 a)) as well as of 25 °C and 3 d (Example 1.3 b)), and dissolved in 200 µl of a 0.1 M sodium phosphate buffer (0.1 M, 9.15 M NaCl, 50 mM EDTA, pH 7.2), 10 µl of a solution of 2.5 µmol AMAS (Sigma Aldrich, Taufkirchen, D) in DMSO were added. The clear solution was incubated for 80 min at 25 °C and 20 min at 40 °C. Remaining AMAS was removed by centrifugal filtra-

tion with a VIVASPIN 0.5 ml concentrator, 5 KD MWCO (VIVASCIENCE, Hannover, D) at 13,000 rpm, washing 4 times and 30 min each with the phosphate buffer.

To the residual solution, 15 µg of ThioEPO as produced according to example 5 (1 µg/µl in phosphate buffer) were added, and the mixture was incubated for 16 h at 25 °C. After lyophilisation, the crude product was analysed by SDS-Page with NuPAGE 10% Bis-Tris Gels/MOPS buffer (Invitrogen, Carlsbad, USA) as described in the instructions given by Invitrogen. The gel is stained with Roti-Blue Coomassie staining reagent (Roth, Karlsruhe, D) overnight.

The experimental result is shown in Fig 4. A successful conjugation is indicated by the migration of the protein band to higher molecular weights. The increased bandwidth is due to the molecular weight distribution of the HES derivatives used and the number of HES derivatives linked to the protein.

#### **Example 7      Preparative production of HES-EPO conjugates**

##### **Summary**

HES-EPO conjugates were synthesized by coupling of HES derivatives (average mw of 18,000 Dalton; hydroxyethyl substitution degree of 0.4) to the partially (mild periodate) oxidized sialic acid residues on the oligosaccharide chains of recombinant human EPO. Based on carbohydrate structural analysis the modifications introduced did not affect the structural integrity of the core oligosaccharide chains since MALDI/TOF-MS of the mild acid treated HES-modified glycans revealed intact neutral N-acetyllactosamine-type chains which were indistinguishable from those observed in unmodified EPO product. The results obtained indicate that at least 3 modified HES-residues are attached per EPO molecule in the case of the EPO preparation which was subjected to modification without prior partial sialic acid removal. An EPO variant lacking about 50% of the sialic acid residues of the former protein showed a similar apparent high molecular weight mobility in SDS-PAGE (60-110

KDa vs 40 KDa for the BRP EPO standard). The HES modified EPO is stable under standard ion-exchange chromatography conditions at room temperature at pH 3-10.

5 The EPO-bioassay in the normocythaemic mouse system indicates that the HES-modified EPO has 2.5-3.0 fold higher specific activity (IU/mg) in this assay when compared to the International BRP EPO reference standard based on protein determination using the UV absorption value from the European Pharmacopeia and an RP-HPLC EPO protein determination method calibrated against the BRP EPO standard preparation.

10

### **Example 7.1     Materials and methods**

#### **(a)   Liberation of N-linked oligosaccharides by digestion with N-glycosidase**

15 Samples were incubated with 25 units (according to manufacturer's specification, Roche Diagnostics, Germany) of recombinant PNGase F over night at 37°C. Complete digestion was monitored by the specific mobility shift of the protein in SDS-PAGE. The released N-glycans were separated from the polypeptide by addition of 3 volumes of cold 100% ethanol and incubation at -20°C for at least 2 hours (Schroeter  
20 S et al., 1999). The precipitated protein was removed by centrifugation for 10 minutes at 4°C at 13000 rpm. The pellet was then subjected to two additional washes with 500 µl of ice-cold 75% ethanol. The oligosaccharides in the pooled supernatants were dried in a vacuum centrifuge (Speed Vac concentrator, Savant Instruments Inc., USA). The glycan samples were desalted using Hypercarb cartridges (25 mg or 100  
25 mg of HyperCarb) as follows prior to use: the columns were washed with 3 x 500 µl of 80% acetonitrile (v/v) in 0.1% TFA followed by washes with 3 x 500 µl of water. The samples were diluted with water to a final volume of 300 µl – 600 µl before loading onto the cartridge which then was rigorously washed with water. Oligosaccharides were eluted with 1.2 ml (25 mg cartridges; 1.8 ml in the case of 100 mg  
30 cartridges) 25% acetonitrile in water containing 0.1% trifluoroacetic acid (v/v). The eluted oligosaccharides were neutralized with 2 M NH<sub>4</sub>OH and were dried in a Speed Vac concentrator. In some cases desalting of N-glycosidase released oligosaccha-



rides was performed by adsorption of the digestion mixture from samples < 100 µg of total (glyco)protein onto 100 mg Hypercarb cartridges.

**(b) Analysis of oligosaccharides by matrix-assisted laser desorption/ ionization  
5 time-of-flight mass-spectrometry (MALDI/TOF/TOF-MS)**

A Bruker ULTRAFLEX time-of-flight (TOF/TOF) instrument was used: native desialylated oligosaccharides were analyzed using 2,5-dihydroxybenzoic acid as UV-absorbing material in the positive as well as in the negative ion mode using the re-  
10 flectron in both cases. For MS-MS analyses, selected parent ions were subjected to laser induced dissociation (LID) and the resulting fragment ions separated by the second TOF stage (LIFT) of the instrument. Sample solutions of 1 µl and an approximate concentration of 1-10 pmol·µl<sup>-1</sup> were mixed with equal amounts of the respective matrix. This mixture was spotted onto a stainless steel target and dried at  
15 room temperature before analysis.

**Example 7.2 Preparation and characterization of recombinant human EPO  
(EPO-GT-1)**

20 EPO was expressed from recombinant CHO cells as described (Mueller PP et al., 1999, Dorner AJ et al., 1984) and the preparations were characterized according to methods described in the Eur. Phar. (*Ph. Eur. 4, Monography 01/2002:1316: Erythropoietin concentrated solution*). The final product had a sialic acid content of 12 nMol (+/- 1.5 nMol) per nMol of protein. The structures of N-linked oligosaccha-  
25 rides were determined by HPAEC-PAD and by MALDI/TOF-MS as described (Nimtz et al., 1999, Grabenhorst, 1999). The EPO preparations that were obtained contained di-, tri- and tetrasialylated oligosaccharides (2-12%, 15-28% and 60-80%, respectively, sulphated and pentasialylated chains were present in small amounts). The overall glycosylation characteristics of EPO preparations were similar to that of  
30 the international BRP EPO standard preparation.

The isoelectric focusing pattern of the recombinant EPO was comparable to that of the international BRP Reference EPO standard preparation showing the corresponding isoforms. 25% of the EPO protein lacked O-glycosylation at Ser<sub>126</sub> of the polypeptide chain.

5

### **Example 7.3      Preparation of partially desialylated EPO forms**

EPO GT-1 protein (2.84 mg/ml) was heated to 80°C in 20 mM Na-phosphate buffer pH 7.0 and then 100 µl of 1 N H<sub>2</sub>SO<sub>4</sub> was added per 1 ml of the EPO solution; incubation was continued for 5 min, 10 min and 60 min, respectively, yielding EPO preparations of different degree of sialylation. Quantitation of oligosaccharides with 0-4 sialic acids was performed after liberation of oligosaccharides with polypeptide N-glycosidase and isolation of N-linked chains was performed by desalting using Hypercarb cartridges (25 mg HyperSep Hypercarb; ThermoHypersil-Keystone, UK).  
EPO preparations were neutralized by addition of 1 N NaOH and were frozen in liquid N<sub>2</sub> and were stored at -20°C until further use.

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### **Example 7.4      Periodate oxidation of sialylated EPO forms**

To 10 mg of untreated or mild acid treated EPO dissolved in 3.5 ml of 20 mM Na-phosphate buffer pH 7.0 was added 1.5 ml of 0.1 M Na-acetate buffer pH 5.5 and the mixture was cooled to 0°C in an ice-bath; 500 µl of 10 mM Na-periodate was added and the reaction mixture was kept in the dark for 60 min at 0°C. Then 10 µl of glycerol was added and incubation was continued for further 10 min in the dark. The partially oxidized EPO forms were separated from reagents by desalting using VIVASPIN concentrators (10,000 MWCO, PES Vivascience AG, Hannover, Germany) according to manufacturer's recommendation at 3000 rpm in a laboratory centrifuge equipped with a fixed angle rotor. After freezing in liquid nitrogen the EPO preparations were stored in a final volume of 4 ml at -20°C.

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25  
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100 µg aliquots of the partially oxidized EPO preparation were subjected to N-glycosidase treatment and oligosaccharides were isolated using Hypercarb cartridges

as described. Oligosaccharides were desialylated by mild acid treatment and were analyzed by HPAEC-PAD and their retention times were compared to those of authentic standard oligosaccharides as described (Nimtz et al., 1990 and 1993).

#### 5    **Example 7.5    Reduction of EPO disulfides with dithioerythritol**

5 mg of EPO-GT-1 was incubated in 5 ml of 0.1 M Tris/HCl buffer pH 8.1 in the presence of 30 mM dithioerythritol (DTT) at 37°C for 60 minutes; removal of DTT was achieved by using a Vivaspin concentrator at 4 °C, 4 cycles of buffer exchange.  
10    The final reduced EPO preparation was frozen in liquid nitrogen and stored at -20°C in 50 mM Na-acetate buffer pH 5.5.

#### **Example 7.6    EPO protein determination**

15    Quantitative determination of EPO protein was performed by measuring UV absorption at 280 nm according to the Eur. Phar. (European Pharmacopeia 4, Monography 01/2002: 1316: erythropoietin concentrated solution) in a cuvette with 1 cm path length. In addition, EPO was quantitated by applying a RP-HPLC method using a RP-C4 column (Vydac Protein C4, Cat.# 214TP5410, Grace Vydac, Ca, US); the  
20    HPLC method was calibrated using the erythropoietin BRP 1 reference standard (European Pharmacopeia, Conseil de l'Europe B.P. 907-F67029, Strasbourg Cedex 1).

#### **Example 7.7    Oxidation of desialylated EPO with galactose oxidase**

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4.485 mg of completely desialylated EPO was incubated in 20 mM Na-phosphate buffer pH 6.8 in the presence of 16 µl catalase (6214 units/200 ml) and 80 µl of galactose oxidase (2250 units/ml from *Dactylium dendroides* (Sigma-Aldrich, Steinheim, Germany); incubation at 37°C was over night; 2 times 20 µl of galactose oxidase was added after 4 hours and after 8 hours after starting of the incubation.  
30

#### **Example 7.8    Preparation of EPO samples for bioassays**

### *Purification of EPO from incubations of periodate- or galactose-oxidase-oxidized EPO protein preparations with activated HES*

5 Purification of EPO samples (removal of unreacted HES derivatives) was carried out at room temperature. The EPO incubation mixtures (approximately 5 mg of EPO protein) were diluted 1:10 with buffer A (20 mM N-morpholine propane sulfonic acid [MOPS/NaOH] in H<sub>2</sub>O bidest, pH 8.0) and were applied to a column containing 3 ml Q-Sepharose HP (Pharmacia Code no. 17-1014-03, Lot no. 220211) equili-  
10 brated with 10 column volumes (CV) of buffer A by using a flow rate of 0.5 ml/min. The column was washed with 6-8 CV of buffer A (flow rate = 0.8 ml/min) and elution was performed by using buffer B (20 mM morpholine ethane sulfonic acid [MES/NaOH], 0.5 M NaCl in H<sub>2</sub>O bidest, pH 6.5) at a flow rate of 0.5 ml/min. EPO was detected by UV absorption at 280 nm and eluted in about 6 ml. The column was  
15 regenerated by using 3 CV of buffer C (20 mM MES, 1.5 M NaCl in H<sub>2</sub>O adjusted to pH 6.5) and was re-equilibrated by using 10 CV of buffer A (flow rate = 0.7 ml/min).

Buffer exchange of EPO eluates obtained from the Q-Sepharose step was performed using Vivaspin concentrators and phosphate buffered saline (PBS) with each 3 centrifugation cycles per sample; samples were adjusted to 2 ml with PBS and were stored at -20°C.

Only <25% of the partially desialylated and subsequently mild periodate oxidized EPO forms that were subjected to HES-modification were obtained from the Q-Sepharose eluate since under the conditions employed the basic EPO forms did not bind Q-Sepharose and were found in the flow-through together with nonreacted HES derivatives.

30 **Example 7.9** High-pH anion-exchange chromatography with pulsed amp-  
erometric detection (HPAEC-PAD)

Purified native and desialylated oligosaccharides were analyzed by high-pH anion-exchange (HPAE) chromatography using a Dionex BioLC system (Dionex, USA) equipped with a CarboPac PA1 column (0.4 x 25 cm) in combination with a pulsed amperometric detector (PAD) (Schröter et al., 1999; Nimtz et al., 1999). Detector potentials (E) and pulse durations (T) were: E1: +50 mV, T1: 480 ms; E2: +500 mV, T2: 120 ms; E3: -500 mV, T3: 60 ms, and the output range was 500-1500 nA. The oligosaccharides were then injected onto the CarboPac PA1 column which was equilibrated with 100% solvent A. For desialylated oligosaccharides elution (flow rate: 1 ml·min<sup>-1</sup>) was performed by applying a linear gradient (0-20%) of solvent B over a period of 40 min followed by a linear increase from 20-100% solvent B over 5 min. Solvent A was 0.2 M NaOH in bidistilled H<sub>2</sub>O, solvent B consisted of 0.6 M NaOAc in solvent A. For native oligosaccharides the column was equilibrated with 100% solvent C (0.1 M NaOH in bidistilled H<sub>2</sub>O) and elution (flow rate: 1 ml·min<sup>-1</sup>) was performed by applying a linear gradient (0-35%) of solvent D over a period of 48 min followed by a linear increase from 35-100% solvent D over 10 min. Solvent D consisted of 0.6 M NaAc in solvent C.

**Example 7.10 Monosaccharide compositional analysis of N-glycans, HES-modified N-glycans and EPO protein by GC-MS**

20

Monosaccharides were analyzed as the corresponding methyl glycosides after methanolysis, *N*-reacetylation and trimethylsilylation by GC/MS [Chaplin, M.F. (1982) A rapid and sensitive method for the analysis of carbohydrate. *Anal. Biochem.* 123, 336-341]. The analyses were performed on a Finnigan GCQ ion trap mass spectrometer (Finnigan MAT corp., San Jose, CA) running in the positive ion EI mode equipped with a 30 m DB5 capillary column. Temperature program: 2 min isotherm at 80°C, then 10 degrees min<sup>-1</sup> to 300°C.

25

Monosaccharides were identified by their retention time and characteristic fragmentation pattern. The uncorrected results of electronic peak integration were used for quantification. Monosaccharides yielding more than one peak due to anomericity

30

and/or the presence of furanoid and pyranoid forms were quantified by adding all major peaks. 0.5  $\mu$ g of myo-inositol was used as an internal standard compound.

#### **Example 7.11 Results**

5

##### **Example 7.11(a) Characterization of N-glycans of mild acid treated (partially desialylated) EPO-GT-1**

EPO-GT-1 preparations subjected to mild acid treatment for 5, 10 or 60 min. were  
10 analyzed by SDS-PAGE before and after liberation of N-linked oligosaccharides by incubation with N-glycosidase as shown in Figure 5. N-linked oligosaccharides were subjected to HPAEC-PAD oligosaccharide mapping (Figure 6). The untreated EPO-GT-1 contained >90% of N-linked oligosaccharides with 3 or 4 sialic acid residues whereas after 5 min. of incubation in the presence of mild acid <40% of carbohydrate  
15 chains had 3 or 4 sialic acid residues. HPAEC-PAD of the desialylated N-glycans revealed that the ratio of neutral oligosaccharides that were detected for the untreated EPO-GT-1 and remained stable in the preparations subjected to acid treatment for 5, 10 or 60 min. MALDI/TOF-MS of the desialylated glycans revealed that <90% of the proximal fucose was present after mild acid treatment of the protein.

20

##### **Example 7.11(b) Characterization of periodate treated EPO-GT-1**

SDS-PAGE mobility of mild periodate treated EPO forms that were previously subjected to a 5 and 10 minute treatment with acid or were not treated are compared in  
25 Figure 8. The conditions used for periodate oxidation of sialic acids did not change the SDS-PAGE pattern of EPO preparations (compare Fig. 5). Oxidation of sialic acids resulted in a shift of oligosaccharides in HPAEC-PAD analysis to earlier elution times (compare Figures 6 and 9).

30

##### **Example 7.11(c) Characterization of HES-modified EPO derivatives**

**(aa) Time course of HES modification of EPO-GT-1-A with hydroxylamine-modified HES derivative X, produced according to Example 2.4**

400  $\mu$ g of hydroxylamine-modified HES derivative X was added to 20  $\mu$ g of EPO-GT-1-A (mild periodate oxidized EPO, not acid hydrolyzed prior to mild periodate oxidation) in 20  $\mu$ L of 0.5 M NaOAc buffer pH 5.5 and the reaction was stopped after 30 min, 2, 4, and 17 hours, respectively, by freezing samples in liquid nitrogen. Subsequently samples were stored at -20°C until further analysis.

SDS-PAGE sample buffer was added and the samples were heated to 90°C and applied onto SDS-gels. As shown in Figure 10, increasing incubation times resulted in an increased shift towards higher molecular weight of the protein. After 17 hours of incubation in the presence of the hydroxylamine-modified HES derivative X a diffuse Coomassie stained protein band was detected migrating in an area between 60 and 11 KDa, based on the position of molecular weight standards (see left part of Fig. 10). Upon treatment with N-glycosidase most of the protein was shifted towards the position of de-N-glycosylated EPO (see Fig. 10, right gel; arrow A indicates migration position of N-glycosidase, arrow B indicates migration position of de-N-glycosylated EPO; the diffuse protein band visible in the region between the 28 KDa and 36 KDa molecular weight standards presumably represents EPO-forms which are modified by HES and the O-glycosylation site of the molecule. In view of the specificity of N-glycosidase we conclude from this result that in fact HES-modification occurs at the periodate oxidized sialic acid residues of glycans of the EPO protein.

25

**(bb) Characterization of HES-EPO conjugates**

HES-EPO conjugates I (originating from EPO-GT-1 after mild periodate oxidation, i.e. from EPO-GT-1-A), II (resulting from EPO-GT-1 subjected to 5 min acid hydrolysis and mild periodate oxidation), III (resulting from EPO-GT-1 subjected to 10 min acid hydrolysis and mild periodate oxidation) were synthesized as described before. A control incubation (K) was included containing unmodified EPO-GT-1

30

under the same buffer conditions to which an equivalent amount of unmodified HES was added. The incubation mixtures were subjected to further purification for subsequent biochemical analysis of the HES-EPO derivatives.

- 5 Incubations HES-EPO conjugates **I**, **II** and **III** as well as the control incubation **K** were subjected to a Q-Sepharose purification step as described under "Material and Methods" (**Example 7.8**) in order to remove the excess of nonreacted HES-reagent which was expected in flow through of the ion-exchange column. Due to the high amounts of basic EPO forms contained in previously acid treated samples **II** and **III** 10 we expected considerable amounts of modified EPO product from these incubations in the flow through. As is shown in Figure 11, almost all of the EPO material from samples **I** was retained by Q-Sepharose column whereas only approximately 20-30% of the samples **III** and **II** was recovered in the fraction eluting with high salt concentration. All of the protein material from the incubations with HES derivative **X**, both 15 in the flow-through and the fractions eluting with high salt, had apparent higher molecular weight in SDS-PAGE when compared to the control EPO.

- In order to characterize in more detail the HES-modified EPO sample **A** and **K** (see Figure 11) were compared to periodate oxidized form **EPO-GT-1-A**. The samples 20 were subjected to N-glycosidase treatment and as is depicted in Figures 12a and 12b the release of N-glycans resulted in the two low molecular weight bands at the position of the O-glycosylated and nonglycosylated EPO forms of the standard EPO preparation. In the case of sample **A** a further band migrating at the position of the 28 KDa mw standard was detected suggesting HES-modification at the O-glycan of this 25 EPO variant (cf. **Example 7.11(c)(aa)**). This band (and also the heavily HES-modified high mw form of N-glycosylated EPO, see Figs. 12a and 12b) disappeared after subjecting the samples to mild hydrolysis which is in agreement with the view that HES modification was achieved at the periodate oxidised sialic acid residues of erythropoietin.

- 30 Aliquots of the N-glycosidase incubation mixtures were hydrolyzed using conditions enabling the complete removal of sialic acids residues (and also the sialic acid linked



HES derivative) from oligosaccharides; after neutralization, the mixtures were then absorbed onto small Hypercarb columns for their desalting. The columns were washed rigorously with water followed by elution of bound neutral oligosaccharides with 40% acetonitrile in H<sub>2</sub>O containing 0.1% of trifluoroacetic acid. The resulting  
5 oligosaccharides were subjected to MALDI/TOF-MS. The spectra of the desialylated oligosaccharide fractions from sample A, EPO-GT-1-A and sample K showed identical masses for complex type oligosaccharides at  $m/z = 1810$  Da (diantennary),  $2175 =$  triantennary,  $2540 =$  tetraantennary,  $2906 =$  tetraantennary plus 1 N-acetylglucosamine repeat and  $3271 =$  tetraantennary plus 2 N-acetylglucosamine re-  
10 peats; small signals corresponding to lack of fucose (-146) and galactose (minus 162) were detected which are attributable to the acid hydrolysis conditions applied for sialic acid removal (see MALDI-Figures 15, 16 and 17).

In a parallel experiment the N-glycosidase digestion mixture was absorbed onto 1 ml  
15 RP-C18 cartridge (without prior acid hydrolysis of oligosaccharides) and elution was performed with 5% acetonitrile in water containing 0.1% TFA; under these conditions the EPO protein was completely retained onto the RP-material and oligosaccharides were washed off from the column with 5% acetonitrile in H<sub>2</sub>O containing 0.1% TFA. The de-N-glycosylated EPO protein was eluted with 70% acetonitrile in H<sub>2</sub>O  
20 containing 0.1% TFA. The oligosaccharide fractions from the RP-C18 step of N-glycosidase-treated sample A, EPO GT-1-A and sample K were neutralized and subjected to desalting using Hypercarb cartridges as described before. The isolated oligosaccharides were subjected to HPAEC-PAD mapping before (see Figures 13) and after mild acid treatment under conditions which enabled quantitative removal of  
25 sialic acids from glycans (see Figures 14).

The HPAEC-PAD profile for the native material obtained from the HES-modified sample A showed only neglectable signals for oligosaccharides whereas EPO GT-1-A-derived oligosaccharides exhibited the same glycan profile as the one shown in  
30 Fig. 9 (sample named EPO-GT-1 after mild periodate treatment). The elution profile of oligosaccharides obtained from the control EPO sample (K) yielded the expected pattern (compare profile in Figure 6). For comparison, the native oligosaccharide

profile of the international BRP-EPO standard is included for comparison and as reference standard.

5 After mild acid hydrolysis, all oligosaccharide preparations showed an identical elution profile of neutral oligosaccharide structures (see Figures 14) with the expected qualitative and quantitative composition of di-, tri- and tetraantennary complex-type carbohydrate chains as described in the methods section for the EPO preparation which was used as a starting material in the present study. This result demonstrates that the HES-modification of the EPO sample results in a covalent linkage of the  
10 HES derivative which is detached from the EPO-protein by N-glycosidase and is acid-labile since it is removed from the N-glycans using mild acid treatment conditions known to desialylate carbohydrates (see Figures 12a+b).

15 **(cc) Monosaccharide compositional analysis of HES-EPO and HES-EPO N-glycans by GC-MS**

In order to further confirm HES-modification of EPO at the N-glycans of the molecule, EPO samples were digested with N-glycosidase and the EPO protein was adsorbed onto RP-C18 cartridges whereas oligosaccharide material was washed off as  
20 described above. As shown in Table 2, glucose and hydroxyethylated glucose derivatives were detected only in the EPO protein which was subjected to HES-modification at cysteine residues and in oligosaccharide fractions of EPO sample A2.

25 **Example 7.11(d) In-vivo assay of the biological activity of HES-modified EPO**

The EPO-bioassay in the normocythaemic mouse system indicates was performed according to the procedures described in the European Pharmacopeia; the laboratory that carried out the EPO assay was using the International BRP EPO reference standard preparation. For the HES-modified EPO A2 preparation a mean value for the  
30 specific activity of 294,600 units per mg EPO of protein was determined indicating an approximately 3-fold higher specific activity when compared to the International

BRP EPO reference standard preparation that was included in the samples sent for activity assays.

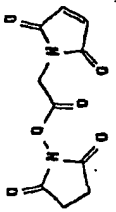
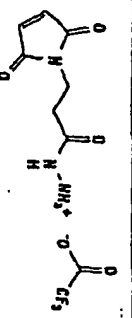
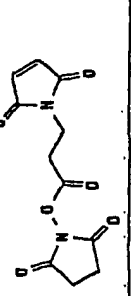
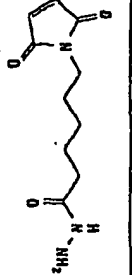
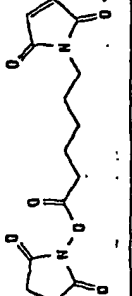
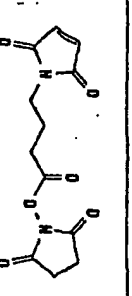
The results of the study are summarized in Table 3.


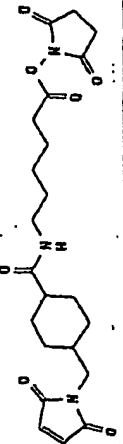
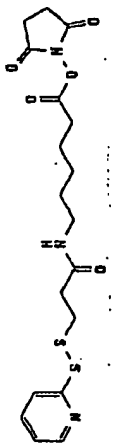
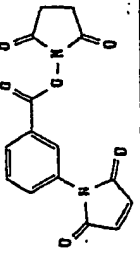

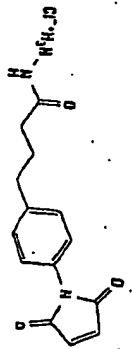
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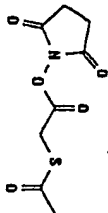
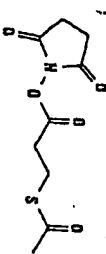
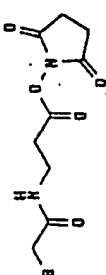
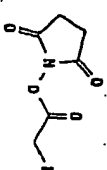
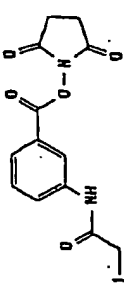
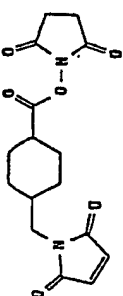
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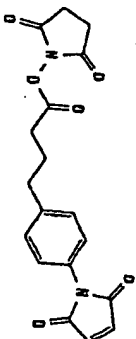

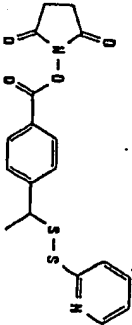
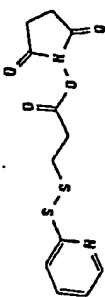
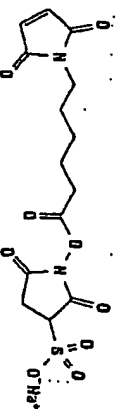
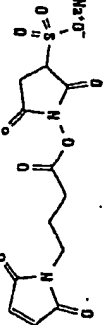
Table 1

- 92 -


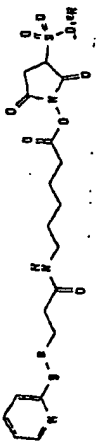
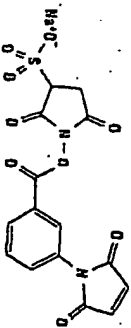
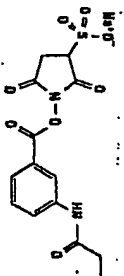
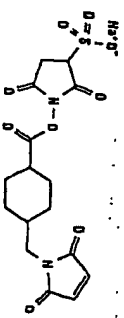
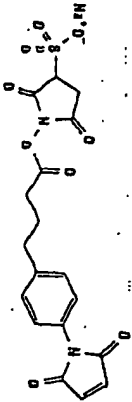
Abreviation	Chemical Name	Type	
AMAS	N-( $\alpha$ -Maleimidocetoxy) succinimide ester	E	
BMPH	N-( $\beta$ -Maleimidopropionic acid) hydrazide·TFA	A	
BMPS	N-( $\beta$ -Maleimidopropoxy) succinimide ester	E	
EMCH	N-( $\epsilon$ -Maleimidocaproic acid) hydrazide	A	
EMCS	N-( $\epsilon$ -Maleimidocaproyloxy) succinimide ester	E	
GMBS	N- $\gamma$ -Maleimidobutyloxy-succinimide ester	E	

Abreviation	Chemical Name	Type	
KMUH	N-( $\kappa$ -Maleimido)undecanoic acid) hydrazide	A	
LC-SMCC	Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxy-(6-amido-caproate)	E	
LC-SPDP	Succinimidyl 6-(3'-[2-pyridyl-dithio]propionamido) hexanoate	F	
MBS	m-Maleimido benzoyl-N-hydroxysuccinimide ester	E	
M <sub>2</sub> C <sub>2</sub> H	4-(N-Maleimidomethyl)-cyclohexane-1-carboxyl-hydrazide·HCl·1/2 dioxane	A	
MPBH	4-(4-N-Maleimidophenyl)-butyric acid hydrazide·HCl	A	


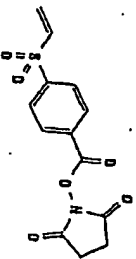
Abbreviation	Chemical Name	Type	
SATA	N-Succinimidyl S-acetylthio-acetate	H	
SATP	N-Succinimidyl S-acetylthio-propionate	H	
SBAP	Succinimidyl 3-(bromoacetamido) propionate	D	
SIA	N-Succinimidyl iodacetate	C	
SIAB	N-Succinimidyl(4-iodoacetyl)amino benzoate	C	
SMCC	Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate	E	

Abbreviation	Chemical Name	Type	
SNPB	Succinimidy 4-(p-maleimidophenyl)butyrate	E	
SMPH	Succinimidy 6-(β-maleimidopropionamido) hexanoate	E	
SMPT	4-Succinimidyloxy-carbonyl-methyl-α-(2-pyridyldithio)toluene	F	
SPDP	N-Succinimidy 3-(2-(2-pyridyldithio)propionate	F	
Sulfo-EMCS	N-(ε-Maleimidocaproyloxy) sulfosuccinimide ester	E	
Sulfo-GMBS	N-γ-Maleimidobutyloxy-sulfosuccinimide ester	E	



Abbreviation	Chemical Name	Type	
Sulfo-KMUS	N-( $\kappa$ -Maleimido undecanoyloxy)-sulfosuccinimide ester	E	
Sulfo-LC-SPDP	Sulfosuccinimidyl 6-(3'-[2-pyridyl]-dithio]propionamido) hexanoate	F	
Sulfo-MBS	m-Maleimido benzoyl-N-hydroxy sulfosuccinimide ester	E	
Sulfo-SIAB	Sulfosuccinimidyl(4-iodoacetyl)aminobenzoate	C	
Sulfo-SMCC	Sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate	E	
Sulfo-SMPB	Sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate	E	

- 97 -

Abbreviation	Chemical Name	Type	
Sulfo-LC-SMPT	Sulfosuccinimidyl 6-( $\alpha$ -methyl- $\alpha$ -[2-pyridylthio]-toluamido)hexanoate	F	
SVSB	N-Succinimidyl-(4-vinylsulfonyl)benzoate	G	

**Table 2**  
**Monosaccharide compositional analysis of glycans from HES-modified EPO**  
**and control samples**

<b>**Mono-saccharide</b>	<b>I. Glycans from A2</b>	<b>II. Glycans from EPO-GT-1A</b>	<b>III. Glycans from K2</b>	<b>III. Glycans from A2</b>	<b>IV. Glycans from EPO- GT-1A</b>	<b>V. Glycans from K2</b>	<b>VI. Cystein modified EPO pro- tein*</b>
fucose	1,935	3,924	2,602	2,246	4,461	2,601	2,181
mannose	6,028	11,020	9,198	6,379	11,668	6,117	6,260
galactose	8,886	19,935	14,427	10,570	16,911	11,555	10,386
glucose	17,968	---	---	21,193	trace	trace	33,021
GlcNAc	7,839	21,310	14,440	11,360	15,953	10,503	10,498
GlcHe1	5,583	---	---	5,926	---	---	14,857
GlcHe2	1,380	---	---	1,552	---	---	3,775
NeuNAc	5,461	822	4,504	3,895	4,871	13,562	13,003
inositol	1,230	2,310	1,620	2,050	1,320	1,134	1,087
<p>* the equivalent of Cys-HES-modified EPO protein was subjected to compositional analysis; the EPO protein was isolated from the HES-incubation mixture by chromatography on a Q-Sepharose column as described above and was desalted by centrifugation using a Vivaspin 5 separation device.</p> <p>** Monosaccharide determinations were performed from single GC runs of the pertrimethylsilylated methylglycosides; the electronical integration values of peaks are given without correction for losses during the derivatisation procedure and recoveries of each compound.</p>							

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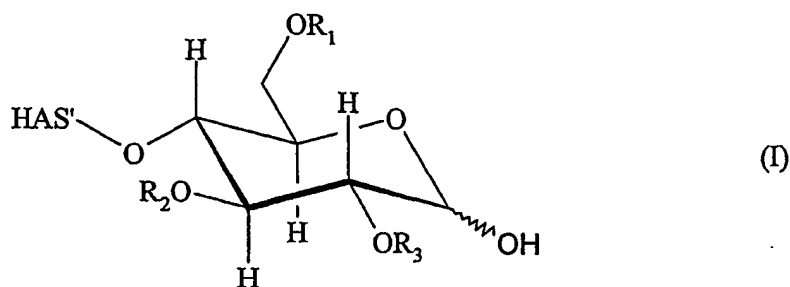
**Table 3**

<b>Sample No.</b>	<b>Sample description</b>	<b>Calculated specific activity of EPO sample</b> (based on A280 nm and RP-HPLC determination)
850247	1. HES-modified EPO A2	344,000 U/mg
850248	2. EPO-GT-1-A	82,268 U/mg
850249	3. Control EPO K2	121,410 U/mg
850250	4. BRP EPO standard	86,702 U/mg
850251	1. diluted with 4 volume of PBS	309,129 U/mg
850252	2. diluted with 4 volume of PBS	94,500 U/mg
850253	3. diluted with 4 volume of PBS	114,100 U/mg
850254	4. diluted with 4 volume of PBS	81,200 U/mg
850255	1. diluted with 4 volume of PBS	230,720 U/mg

5

## Claims

1. A method of producing a hydroxyalkyl starch derivative comprising reacting hydroxyalkyl starch of formula (I)



at its reducing end which is not oxidized prior to said reaction, with a compound of formula (II)

10



wherein  $R_1$ ,  $R_2$  and  $R_3$  are independently hydrogen or a linear or branched hydroxyalkyl group, and wherein either  $R'$  or  $R''$  or  $R'$  and  $R''$  comprise at least one functional group X capable of being reacted with at least one other compound prior to or after the reaction of (I) and (II).

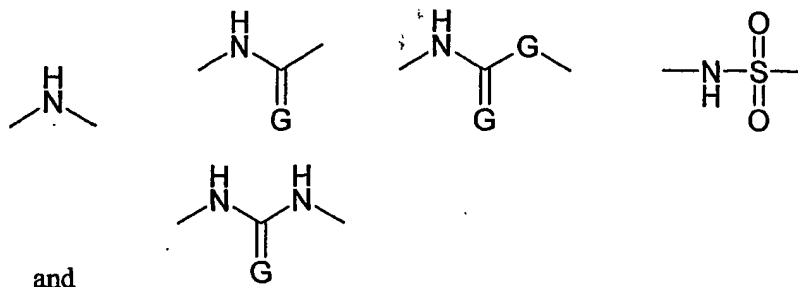
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2. A method as claimed in claim 1 wherein the hydroxyalkyl starch is hydroxyethyl starch.
3. A method as claimed in claim 1 or 2 wherein  $R_1$ ,  $R_2$  and  $R_3$  are independently hydrogen or a 2-hydroxyethyl group.
4. A method as claimed in any of claims 1 to 3 wherein  $R'$  is hydrogen or a linear or branched alkyl or alkoxy group.
5. A method as claimed in claim 4 wherein  $R'$  is hydrogen or a methyl or a methoxy group.

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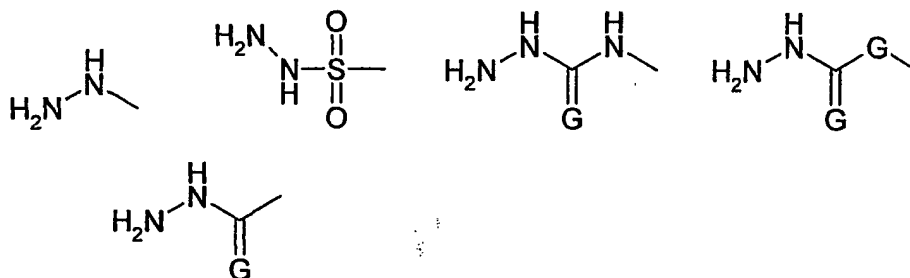
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6. A method as claimed in any of claims 1 to 5 wherein apart from the functional group X, R" comprises at least one additional functional group W which is directly linked to the NH group bridging R' and R", said functional group W being selected from the group consisting of



- 5 where G is O or S and, if present twice, G is independently O or S.

7. A method as claimed in claim 6 wherein, if R' is H, R' and the NH group bridging R' and R" form, together with W, one of following groups:

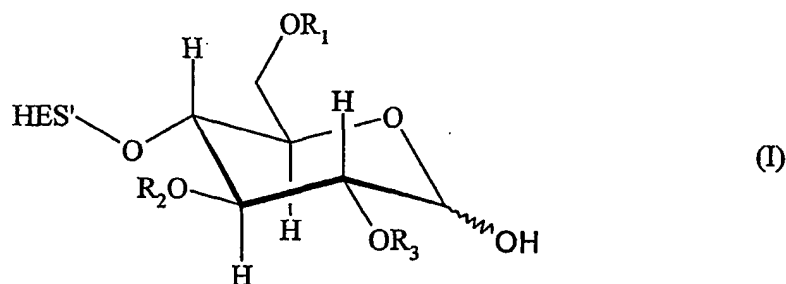


- 10 8. A method as claimed in any of claims 1 to 7 wherein the at least one functional group X is selected from the group consisting of -SH, -NH<sub>2</sub>, -O-NH<sub>2</sub>, -NH-O-alkyl, -(C=G)-NH-NH<sub>2</sub>, -G-(C=G)-NH-NH<sub>2</sub>, -NH-(C=G)-NH-NH<sub>2</sub>, and -SO<sub>2</sub>-NH-NH<sub>2</sub> where G is O or S and, if G is present twice, it is independently O or S.
- 15 9. A method as claimed in any of claims 1 to 6 or 8 wherein the compound according to formula (II) is O-[2-(2-aminoxy-ethoxy)-ethyl]-hydroxyl amine or carbonylhydrazide.
- 20 10. A method as claimed in any of claims 1 to 9 wherein compound (I) is reacted with compound (II) which is not reacted with a further compound prior to the reaction with compound (I).

11. A method as claimed in claim 10 wherein the reaction of compound (I) and compound (II), compound (II) being a hydroxylamine or a hydrazide, is carried out at a temperature of from 5 to 45 °C and at a pH in the range of from 4.5 to 6.5 in an aqueous medium.
12. A method as claimed in claim 10 wherein the reaction of compound (I) and compound (II), said reaction being a reductive amination, is carried out at a temperature of from 25 to 90 °C and at a pH in the range of from 8 to 12 in an aqueous medium.
13. A method as claimed in any claims 10 to 12 wherein the reaction product of compound (I) and compound (II) is reacted with a further compound via the at least one functional group X.
14. A method as claimed in claim 13 wherein the at least one further compound is reacted via a thio group or an oxidized carbohydrate moiety comprised in the at least one further compound.
15. A method as claimed in claim 14 wherein the at least one further compound is a polypeptide.
16. A method as claimed in claim 15 wherein the polypeptide is erythropoietin.
17. A method as claimed in any of claims 13 to 16 wherein the reaction is carried out at a temperature in the range of from 4 to 37 °C.
18. A method as claimed in any of claims 13 to 17 wherein the reaction is carried out in an aqueous medium.
19. A method as claimed in claim 13 wherein the further compound is a crosslinking compound.

20. A method as claimed in claim 19 wherein the reaction product of the reaction product of compounds (I) and (II) with the crosslinking compound is reacted with a second further compound.
- 5 21. A method as claimed in claim 20 wherein the second further compound is a polypeptide.
22. A method as claimed in claim 20 or 21 wherein the second further compound is reacted via a thio group or an oxidized carbohydrate moiety comprised in the at  
10 least one further compound.
23. A method as claimed in claim 13 wherein the further compound is a reaction product of a crosslinking compound and a second further compound.
- 15 24. A method as claimed in claim 23 wherein the second further compound is a polypeptide.
25. A method as claimed in any claims 1 to 3 wherein compound (II) is reacted with a further compound prior to the reaction with compound (I).
- 20 26. A method as claimed in claim 25 wherein the at least one further compound is reacted via a thio group or an oxidized carbohydrate moiety comprised in the at least one further compound.
- 25 27. A method as claimed in claim 25 or 26 wherein the at least one further compound is a polypeptide.
28. A method as claimed in claim 27 wherein the polypeptide is erythropoietin.
- 30 29. A method as claimed in any of claims 25 to 28 wherein the reaction is carried out at a temperature in the range of from 4 to 37 °C.

30. A method as claimed in any of claims 25 to 29 wherein the reaction is carried out in an aqueous medium.
31. A method as claimed in claim 25 wherein the further compound is a crosslinking compound.
32. A method as claimed in claim 31 wherein the crosslinking compound is reacted with a second further compound prior to the reaction with compound (II).
33. A method as claimed in claim 25 wherein the further compound is a reaction product of a crosslinking compound and a second further compound.
34. A method as claimed in claim 33 wherein the second further compound is a polypeptide.
35. A method of producing a hydroxyethyl starch derivative comprising reacting hydroxyethyl starch of formula (I)



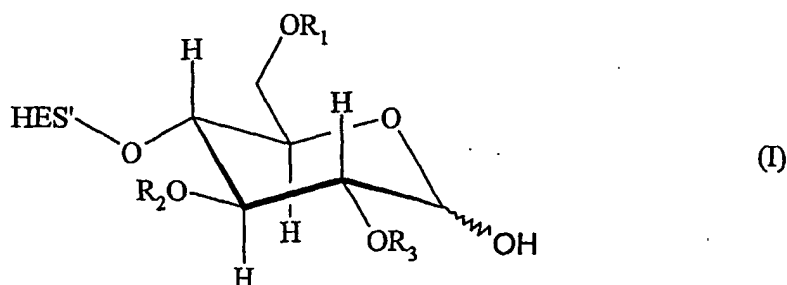
at its reducing end which is not oxidized prior to said reaction, in an aqueous medium with a compound of formula (II)



- wherein  $\text{R}_1$ ,  $\text{R}_2$  and  $\text{R}_3$  are independently hydrogen or a 2-hydroxyethyl group, wherein  $\text{R}'$  is hydrogen or a methyl or a methoxy group, and wherein  $\text{R}''$  comprise at least one functional group X capable of being reacted with at least one other compound prior to or after the reaction of (I) and (II), said functional group X being selected from the group consisting of  $-\text{SH}$ ,  $-\text{NH}_2$ ,  $-\text{O-NH}_2$ ,  $-\text{NH-O-alkyl}$ ,  $-(\text{C}=\text{G})\text{-NH-NH}_2$ ,  $-\text{G}-(\text{C}=\text{G})\text{-NH-NH}_2$ ,  $-\text{NH}-(\text{C}=\text{G})\text{-NH-NH}_2$ , and  $-\text{SO}_2\text{-NH-NH}_2$  where G is O or S and, if G is present twice, it is independently G or S.

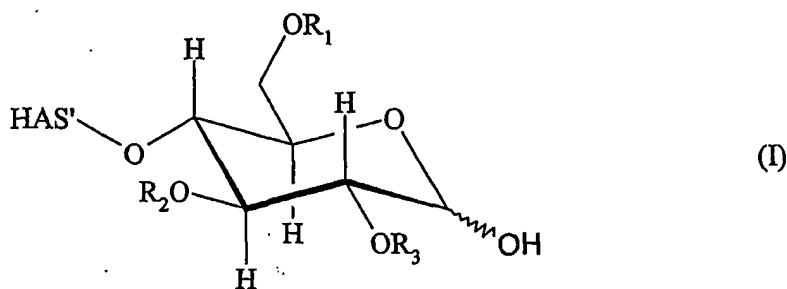


36. A method as claimed in claim 35 wherein the compound according to formula (II) is O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine or carbohydrazide.
37. A method as claimed in claim 35 or 36 wherein the reaction product of compound (I) and compound (II) is reacted in an aqueous medium with a polypeptide via the at least one functional group X comprised in compound (II) and a thio group or an oxidized carbohydrate moiety comprised in the polypeptide.
38. A method as claimed in claim 37 wherein the polypeptide is erythropoietin.
39. A method as claimed in claim 35 wherein compound (II) is reacted in an aqueous medium with a polypeptide via the at least one functional group X comprised in compound (II) and a thio group or an oxidized carbohydrate moiety comprised in the polypeptide, and the resulting reaction product is reacted with compound (I).
40. A method as claimed in claim 39 wherein the polypeptide is erythropoietin.
41. A method of producing a hydroxyethyl starch derivative comprising reacting hydroxyethyl starch of formula (I)



at its reducing end which is not oxidized prior to said reaction, in an aqueous medium with O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine wherein  $R_1$ ,  $R_2$  and  $R_3$  are independently hydrogen or a 2-hydroxyethyl group, and reacting the reaction product in an aqueous medium with erythropoietin via an oxidized carbohydrate moiety comprised in said erythropoietin.

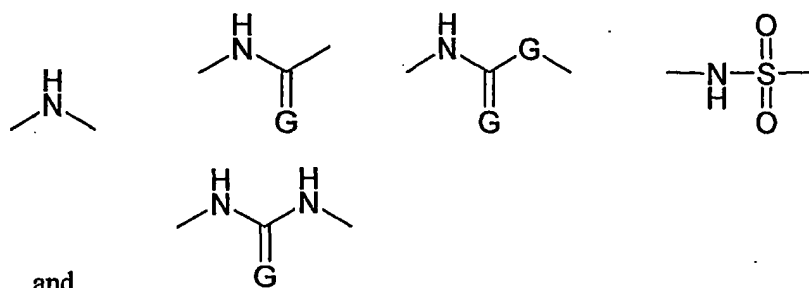
42. A hydroxyalkyl starch derivative obtainable by a method comprising reacting hydroxyalkyl starch (HAS) of formula (I)



at its reducing end which is not oxidized prior to said reaction, with a compound of formula (II)

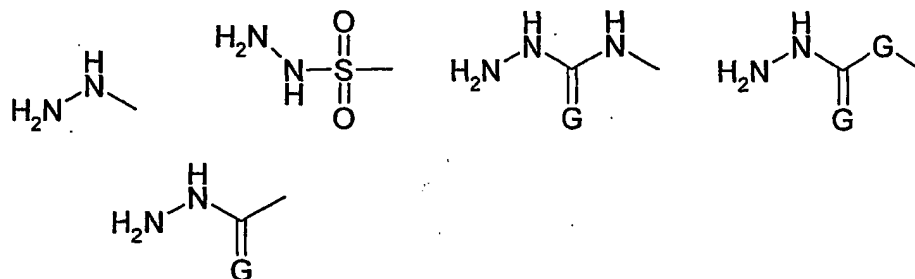


- 5 wherein  $R_1$ ,  $R_2$  and  $R_3$  are independently hydrogen or a linear or branched hydroxyalkyl group, and wherein either  $R'$  or  $R''$  or  $R'$  and  $R''$  comprise at least one functional group X capable of being reacted with at least one other compound prior to or after the reaction of (I) and (II).
- 10 43. A hydroxyalkyl starch derivative as claimed in claim 42 wherein the hydroxyalkyl starch is hydroxyethyl starch.
44. A hydroxyalkyl starch derivative as claimed in claim 42 or 43 wherein  $R_1$ ,  $R_2$  and  $R_3$  are independently hydrogen or a 2-hydroxyethyl group.
- 15 45. A hydroxyalkyl starch derivative as claimed in any of claims 42 to 44 wherein  $R'$  is hydrogen or a linear or branched alkyl or alkoxy group.
46. A hydroxyalkyl starch derivative as claimed in claim 45 wherein  $R'$  is hydrogen or a methyl or a methoxy group.
- 20 47. A hydroxyalkyl starch derivative as claimed in any of claims 42 to 46 wherein apart from the functional group X,  $R''$  comprises at least one additional functional group W which is directly linked to the NH group bridging  $R'$  and  $R''$ , said functional group W being selected from the group consisting of
- 25



where G is O or S and, if present twice, G is independently O or S.

48. A method as claimed in claim 47 wherein, if R' is H, R' and the NH group bridging R' and R'' form, together with W, one of following groups:



5

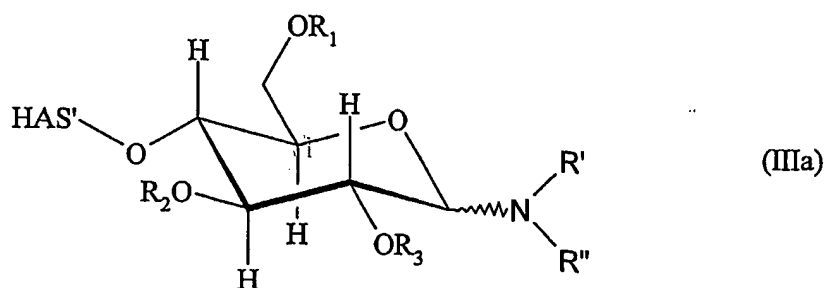
49. A hydroxyalkyl starch derivative as claimed in any of claims 42 to 48 wherein the at least one functional group X is selected from the group consisting of -SH, -NH<sub>2</sub>, -O-NH<sub>2</sub>, -NH-O-alkyl, -(C=G)-NH-NH<sub>2</sub>, -G-(C=G)-NH-NH<sub>2</sub>, -NH-(C=G)-NH-NH<sub>2</sub>, and -SO<sub>2</sub>-NH-NH<sub>2</sub> where G is O or S and, if G is present twice, it is independently G or S.

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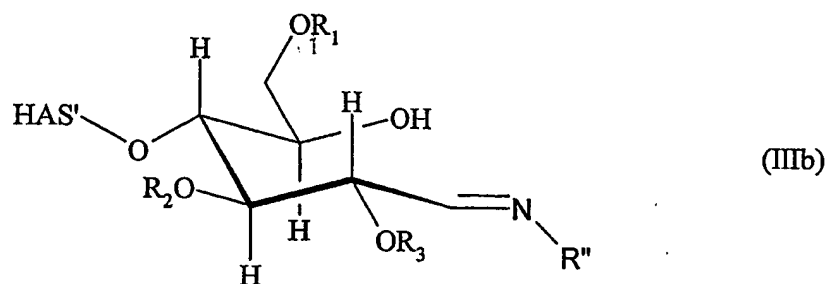
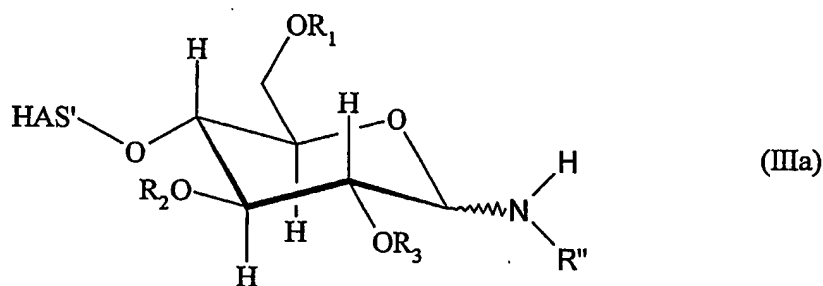
50. A hydroxyalkyl starch derivative as claimed in claim 42 wherein the compound according to formula (II) is O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine or carbonylhydrazide.

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51. A hydroxyalkylstarch derivative as claimed in claim 42 wherein said derivative has a constitution according to formula (IIIa)



52. A hydroxyalkyl starch derivative as claimed in claim 42 wherein R' is hydrogen and wherein the derivative is either a compound of formula (IIIa) or a compound of formula (IIIb) or a mixture of compounds of formulae (IIIa) and (IIIb)



53. A hydroxyalkyl starch derivative as claimed in claim 42 wherein the reaction product of compound (I) with compound (II) is reacted via the at least one functional group X comprised in compound (II) with at least one further compound or wherein compound (II) is reacted via the at least one functional group X with at least one further compound prior to the reaction with compound (I).
54. A hydroxyalkyl starch derivative as claimed in claim 53 wherein the at least one further compound is reacted with compound (II) or with the reaction product of compound (I) and compound (II) via a thio group or an oxidized carbohydrate moiety comprised in the at least one further compound.

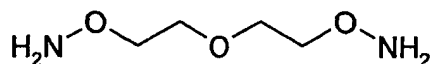
55. A hydroxyalkyl starch derivative as claimed in claim 54 wherein the at least one further compound is a polypeptide.
- 5 56. A hydroxyalkyl starch derivative as claimed in claim 55 wherein the polypeptide is erythropoietin.
57. A hydroxyalkyl starch derivative as claimed in claim 53 wherein the further compound is a crosslinking compound.
- 10 58. A hydroxyalkyl starch derivative as claimed in claim 57 wherein the reaction product of the reaction product of compounds (I) and (II) with the crosslinking compound is reacted with a second further compound.
- 15 59. A hydroxyalkyl starch derivative as claimed in claim 57 wherein the second further compound is a polypeptide.
60. A hydroxyalkyl starch derivative as claimed in claim 58 wherein the second further compound is reacted via a thio group or an oxidized carbohydrate moiety
- 20 comprised in the at least one further compound.
61. A hydroxyalkyl starch derivative as claimed in claim 53 wherein the further compound is a reaction product of a crosslinking compound and a second further compound.
- 25 62. A hydroxyalkyl starch derivative as claimed in claim 61 wherein the second further compound is a polypeptide.
- 30 63. A pharmaceutical composition comprising, in a therapeutically effective amount, a hydroxyalkyl starch derivative as claimed in claim 42 wherein the reaction product of compound (I) with compound (II) is reacted via the at least one functional group X comprised in compound (II) with at least one further compound or wherein compound (II) is reacted via the at least one functional group X with

at least one further compound prior to the reaction with compound (I) and wherein the at least one further compound is a polypeptide.

5 64. A pharmaceutical composition as claimed in claim 63 wherein the polypeptide is reacted with compound (II) or with the reaction product of compound (I) and compound (II) via an oxidized carbohydrate moiety comprised in the polypeptide.

10 65. A pharmaceutical composition as claimed in claim 64 wherein the polypeptide is erythropoietin.

66. A pharmaceutical composition as claimed in claim 65 wherein hydroxyethyl starch is reacted in an aqueous medium with a compound according to the following formula



15 and the reaction product is reacted with erythropoietin.

67. A pharmaceutical composition as claimed in claim 66 wherein the erythropoietin is oxidised with sodium periodate prior to the reaction.

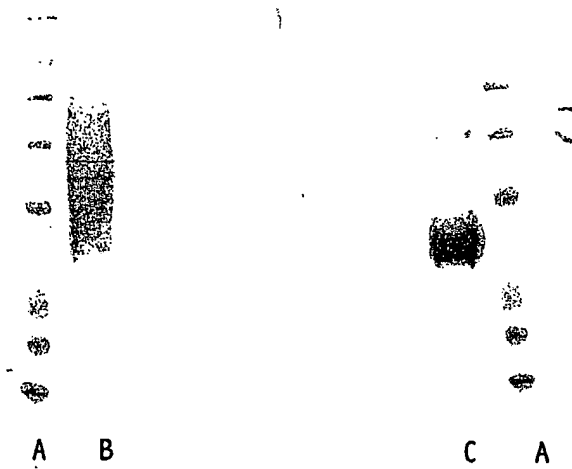
20 68. A pharmaceutical composition as claimed in claim 66 wherein the erythropoietin is partially desialylated and subsequently oxidised with sodium periodate prior to the reaction.

25 69. A pharmaceutical composition comprising, in a therapeutically effective amount, a hydroxyalkyl starch derivative as claimed in claim 42 wherein the reaction product of compound (I) with compound (II) is reacted via the at least one functional group X comprised in compound (II) with at least one further compound or wherein compound (II) is reacted via the at least one functional group X with at least one further compound prior to the reaction with compound (I) and  
30 wherein the at least one further compound is a crosslinking compound and the

reaction product of the reaction product of compounds (I) and (II) with the crosslinking compound is reacted with a polypeptide.

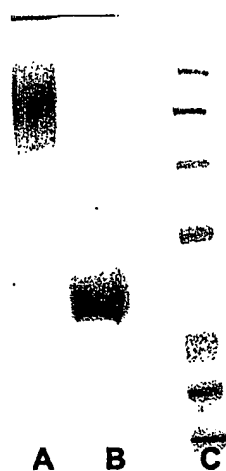
70. A pharmaceutical composition as claimed in claim 69 wherein the polypeptide is erythropoietin.
- 5

**Fig. 1**

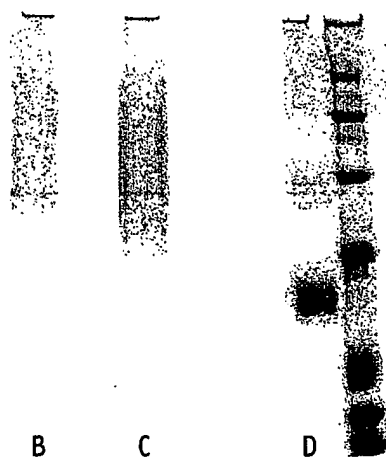




**Fig. 2**



**Fig. 3**



**Fig. 4**

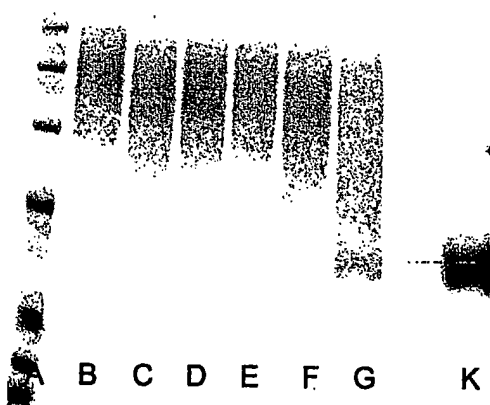


Fig. 5

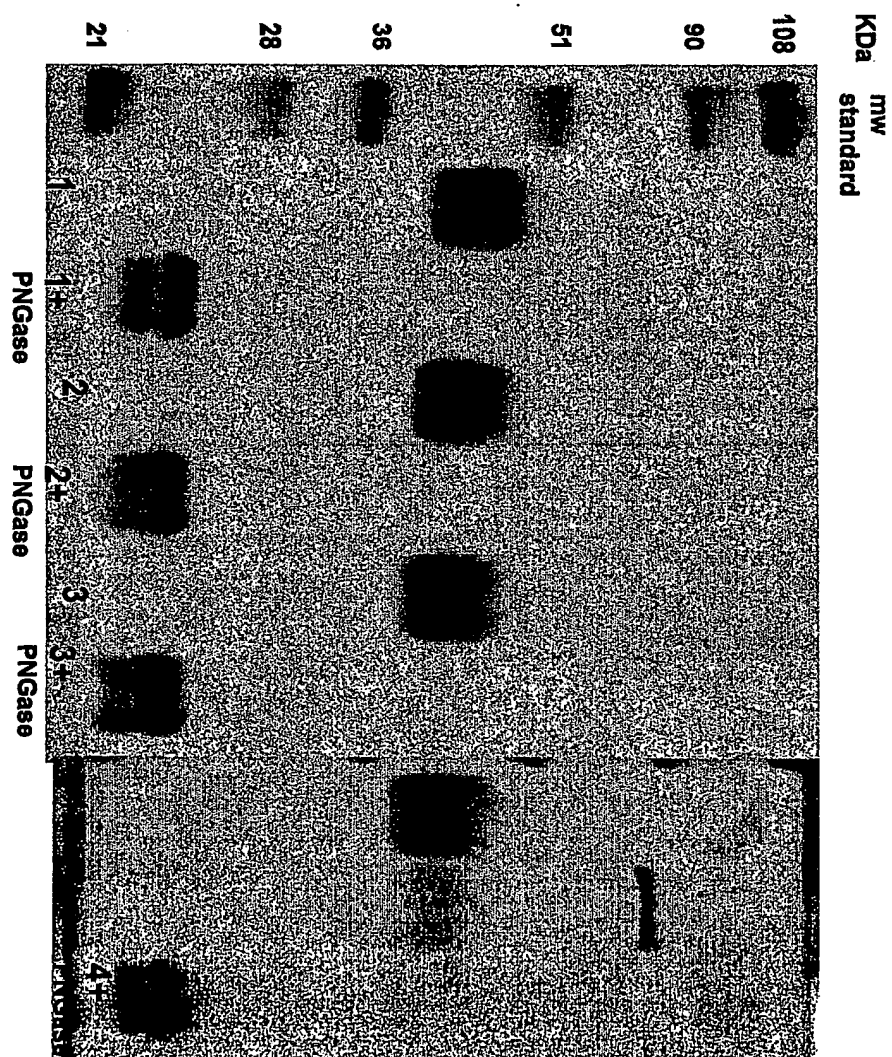
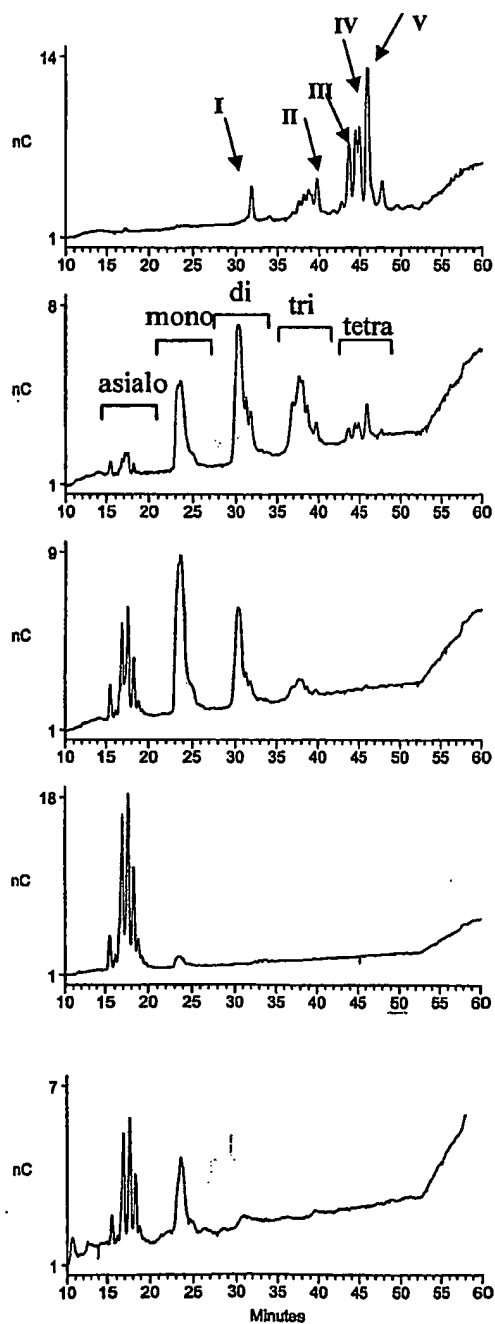


Fig. 6



EPO GT-1  
untreated

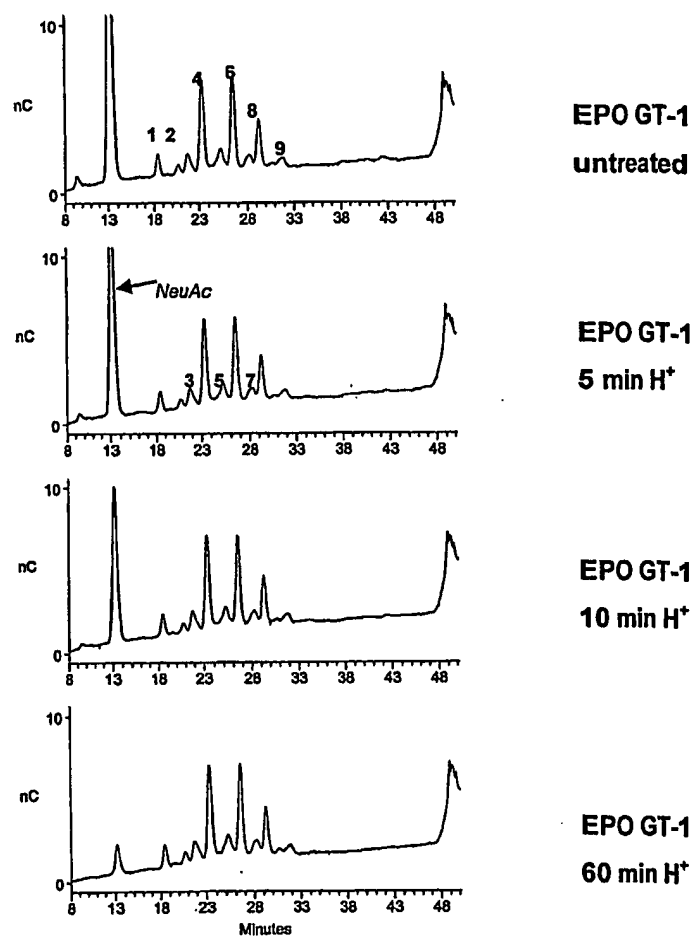
EPO GT-1  
5 min H<sup>+</sup>

EPO GT-1  
10 min H<sup>+</sup>

EPO GT-1  
60 min H<sup>+</sup>

EPO GT-1  
60 min H<sup>+</sup>  
+ Gal oxidase

Fig. 7



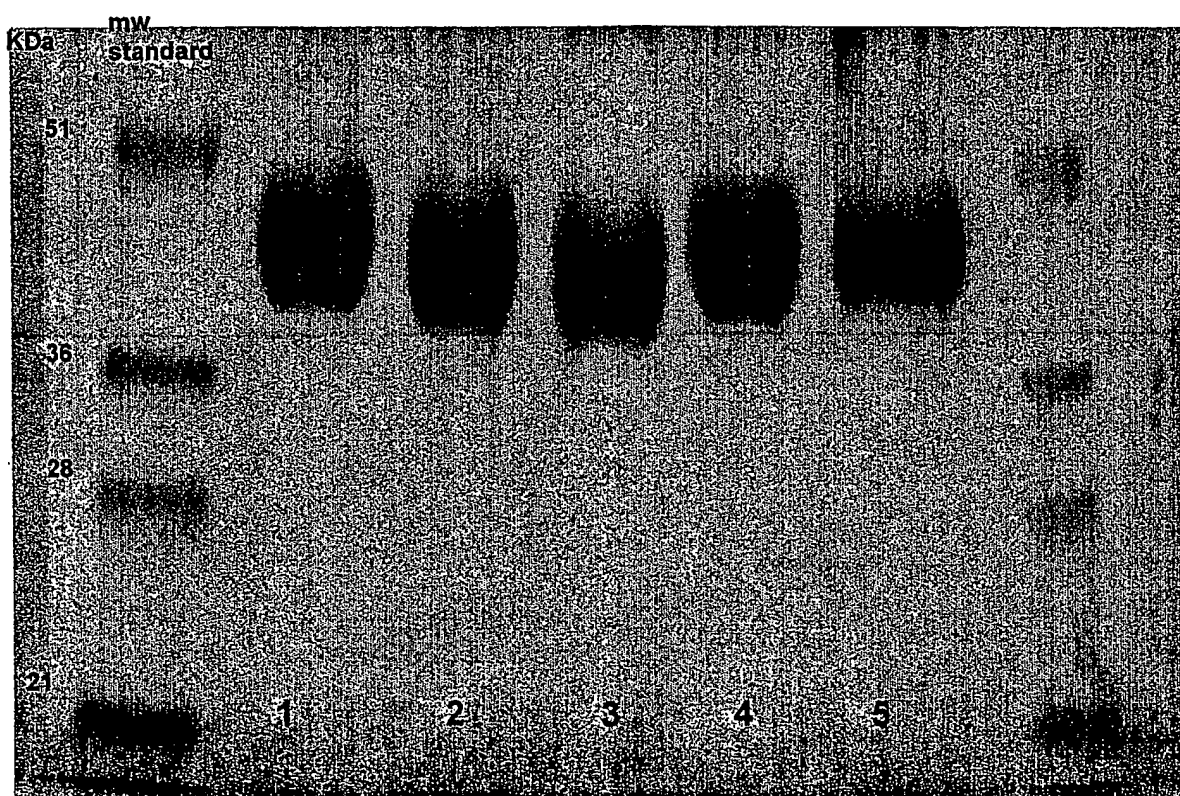
**Fig. 8**

Fig. 9

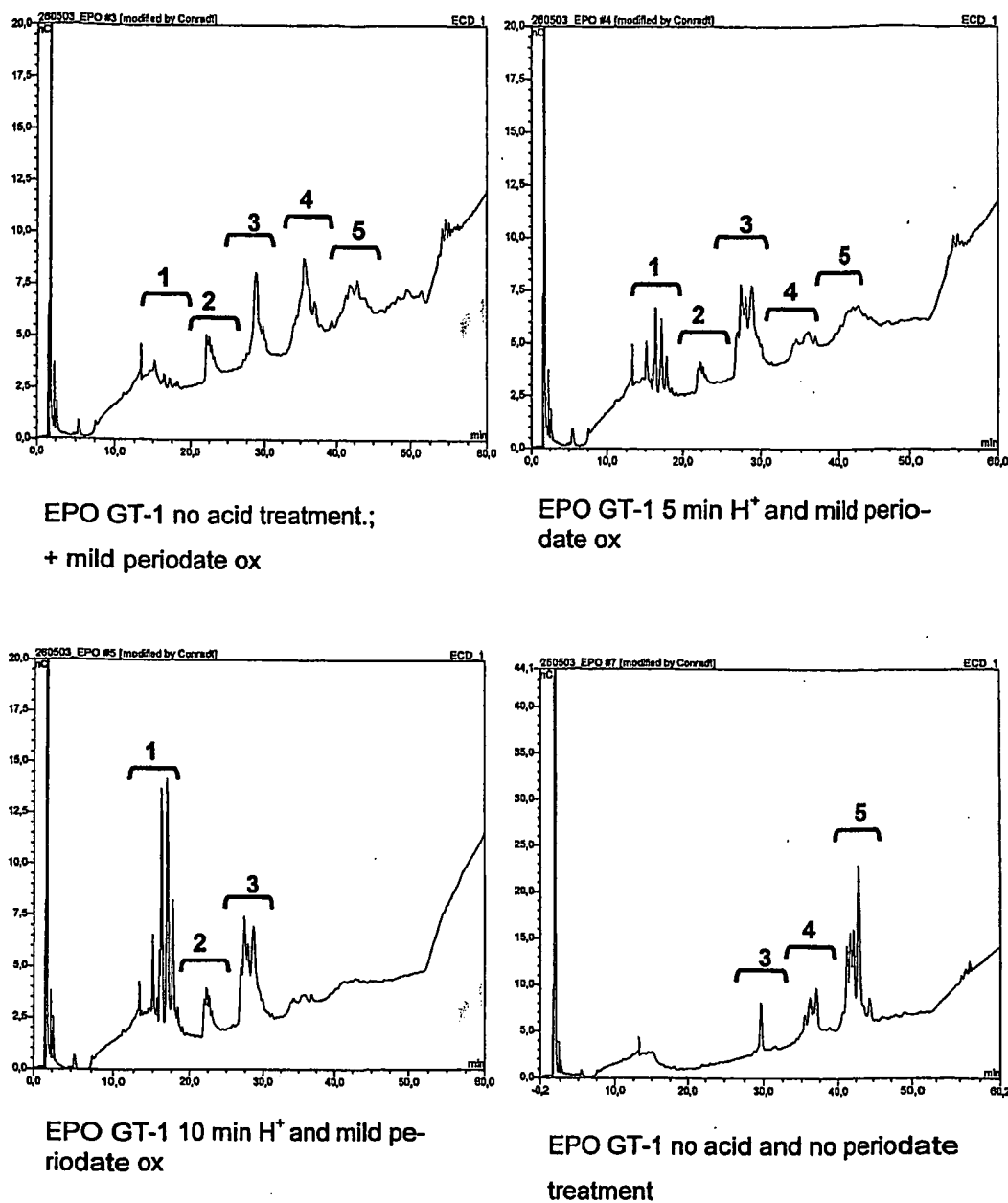
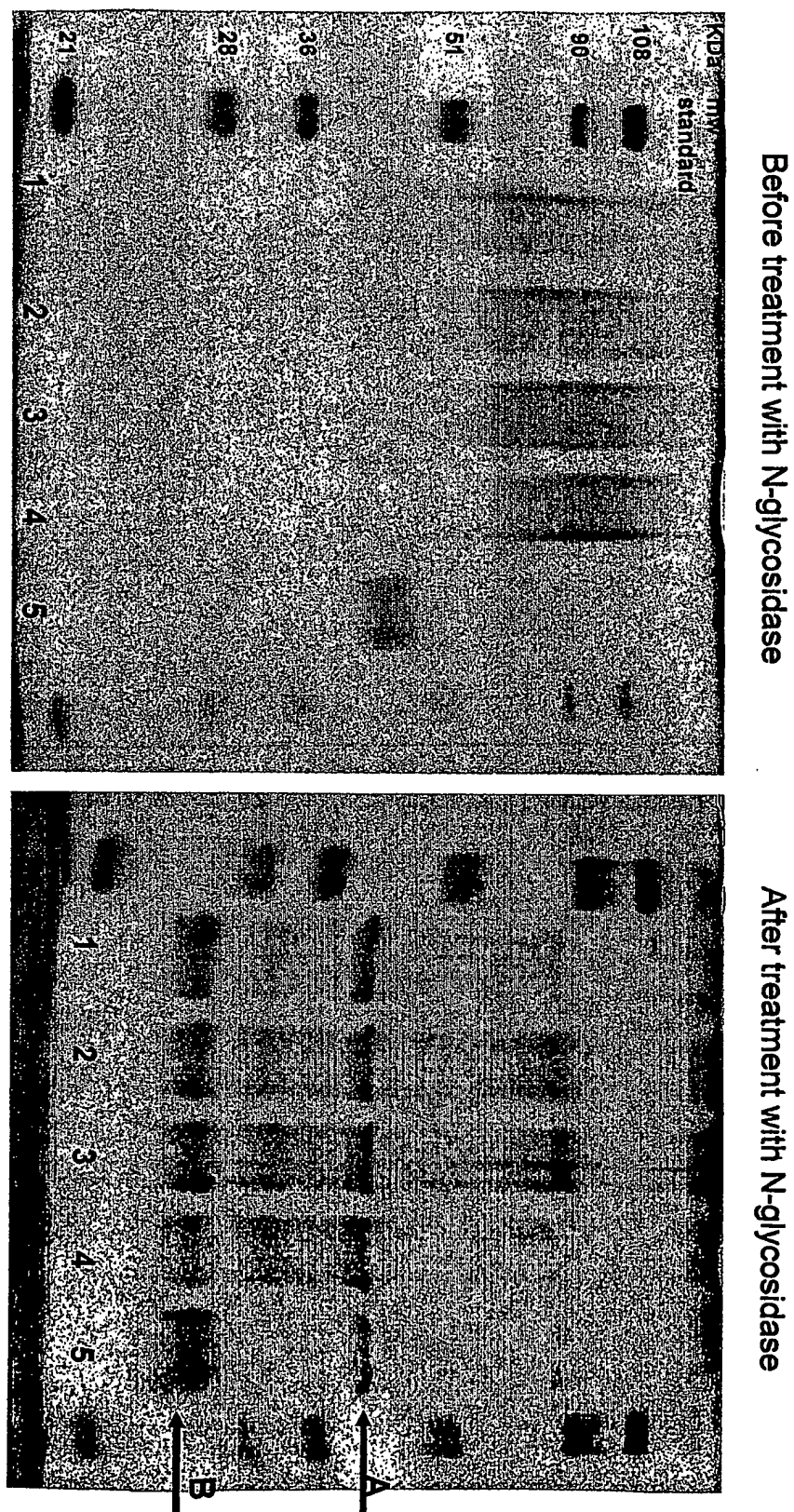




Fig. 10



**Fig. 11**

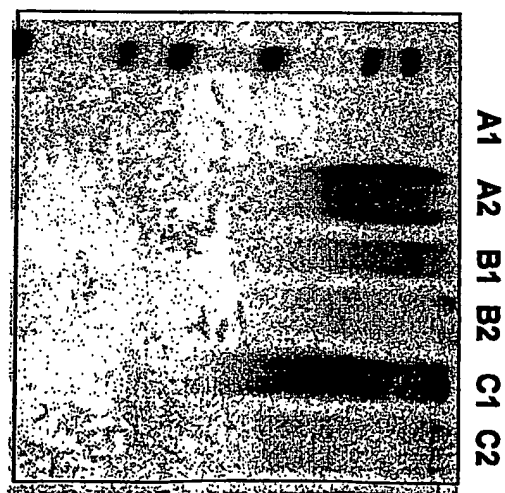
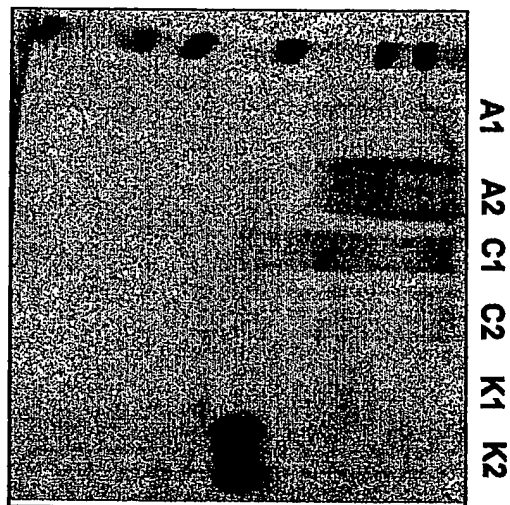
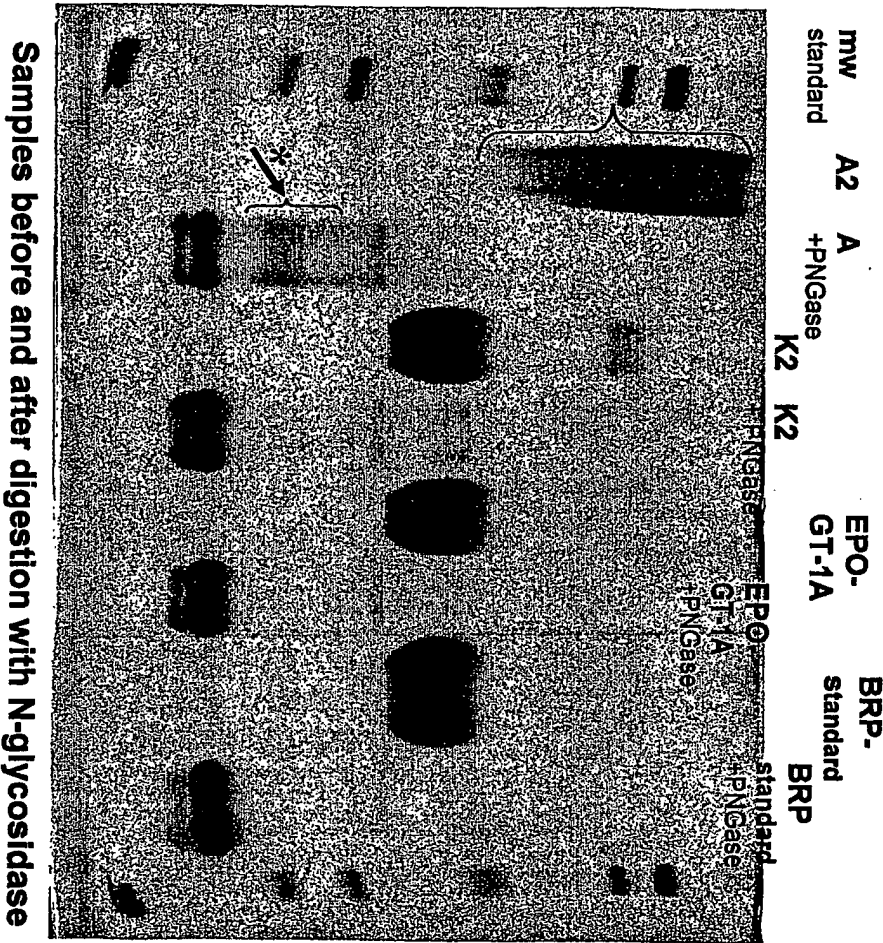


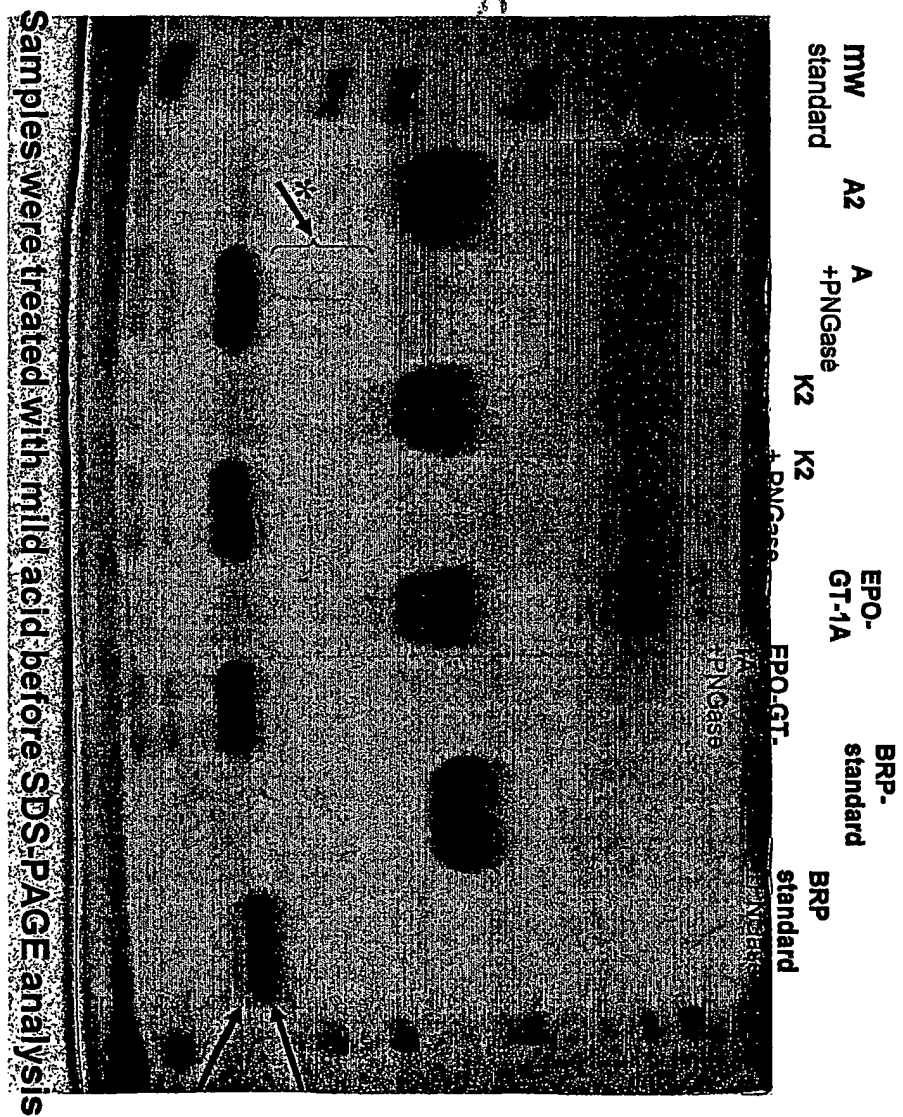
Fig. 12a



Samples before and after digestion with N-glycosidase

O-glycosylated EPO  
non-glycosylated EPO

Fig. 12b



Samples were treated with mild acid before SDS-PAGE analysis

O-glycosylated EPO  
non-glycosylated EPO

Fig. 13

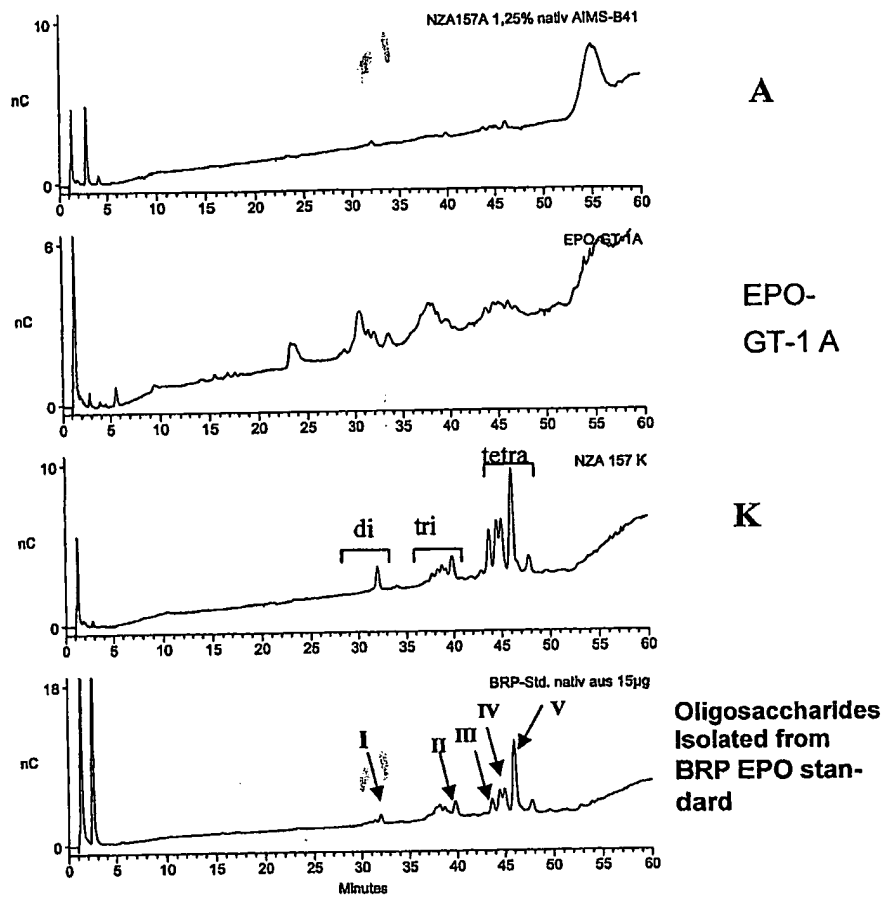


Fig. 14

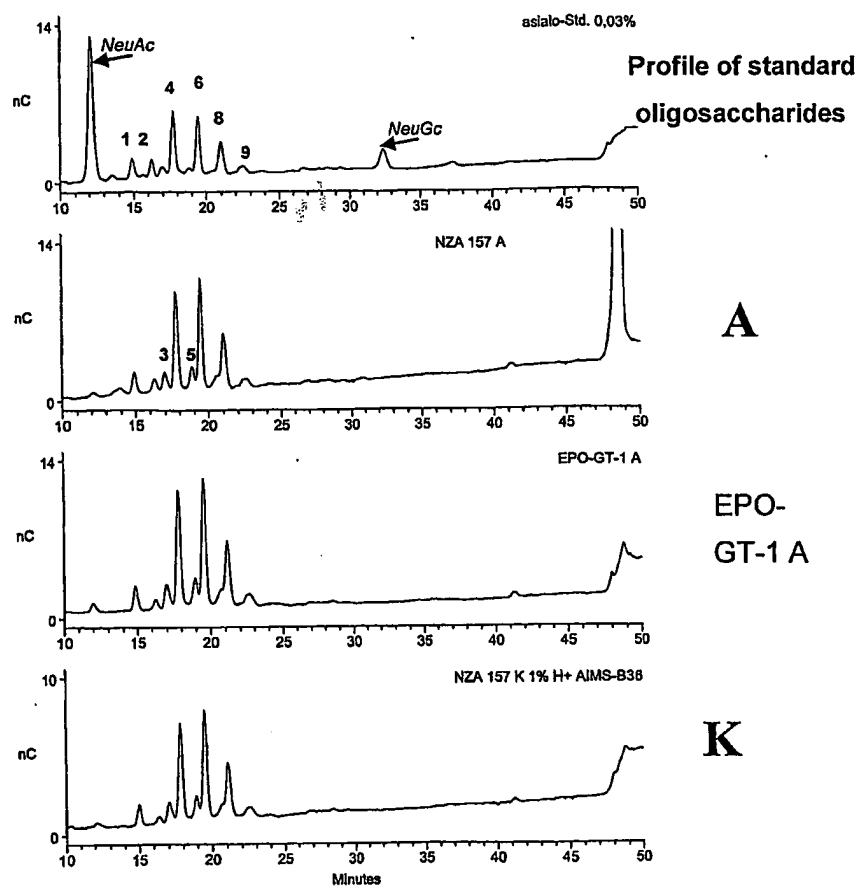


Fig. 15

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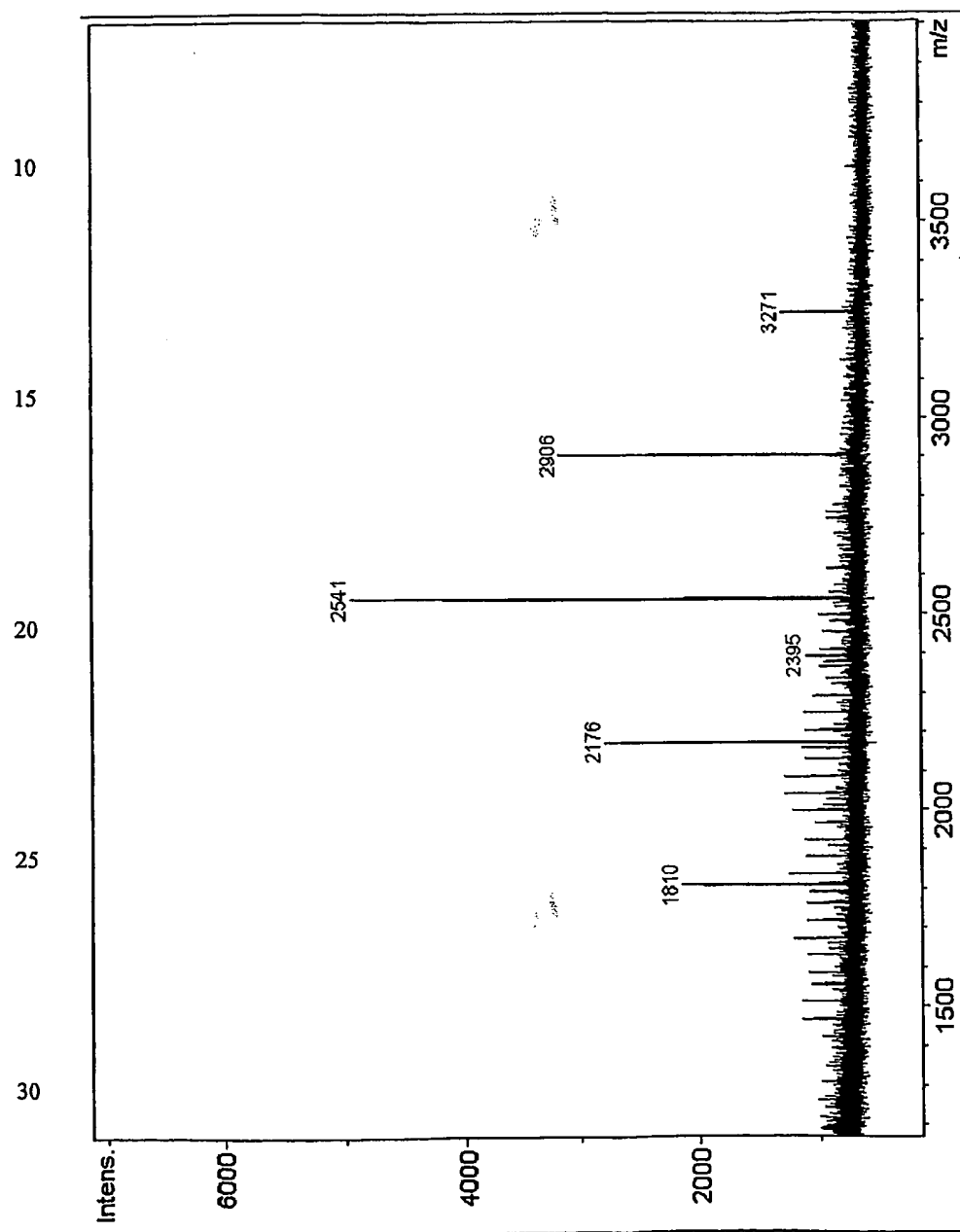


Fig. 16

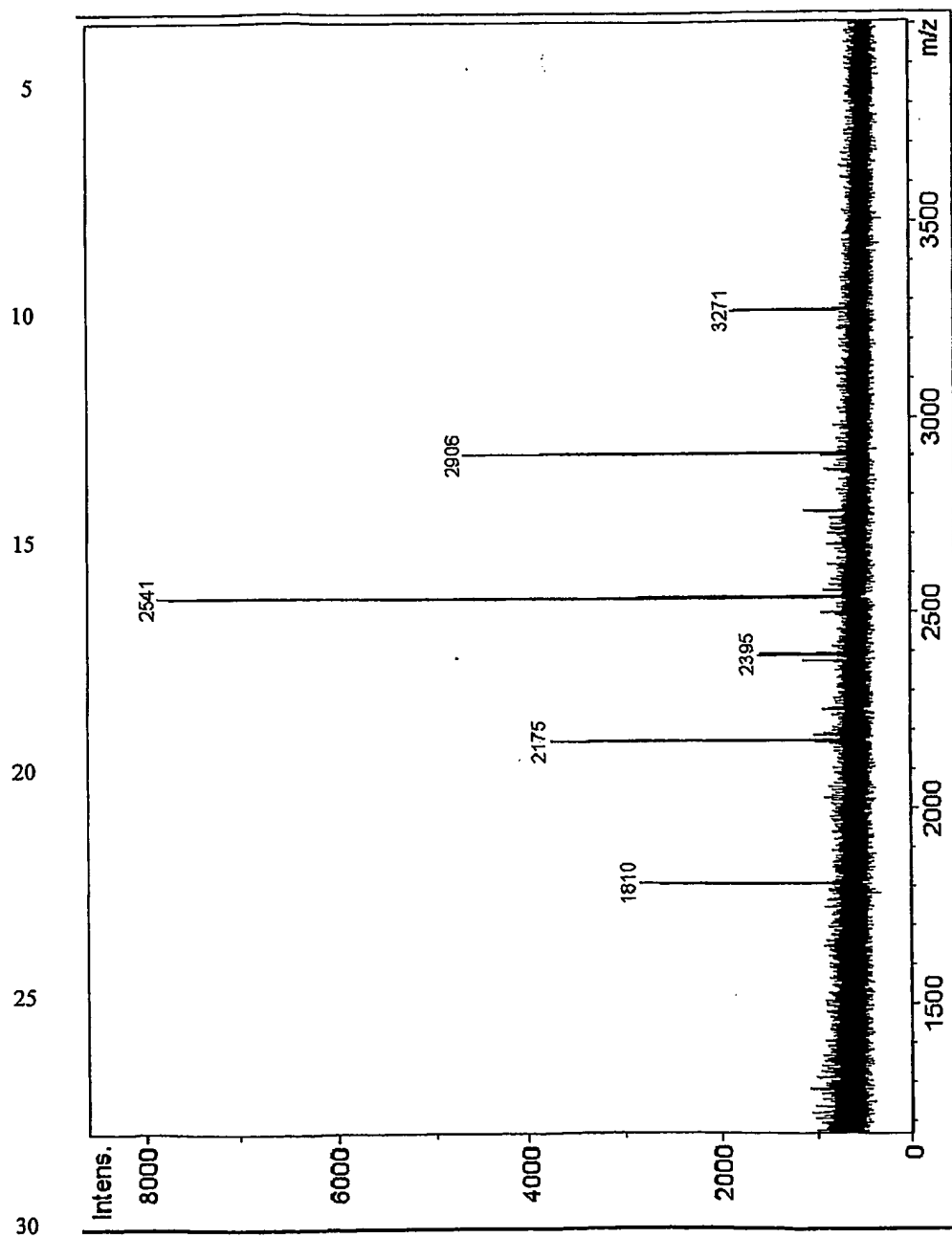




Fig. 17

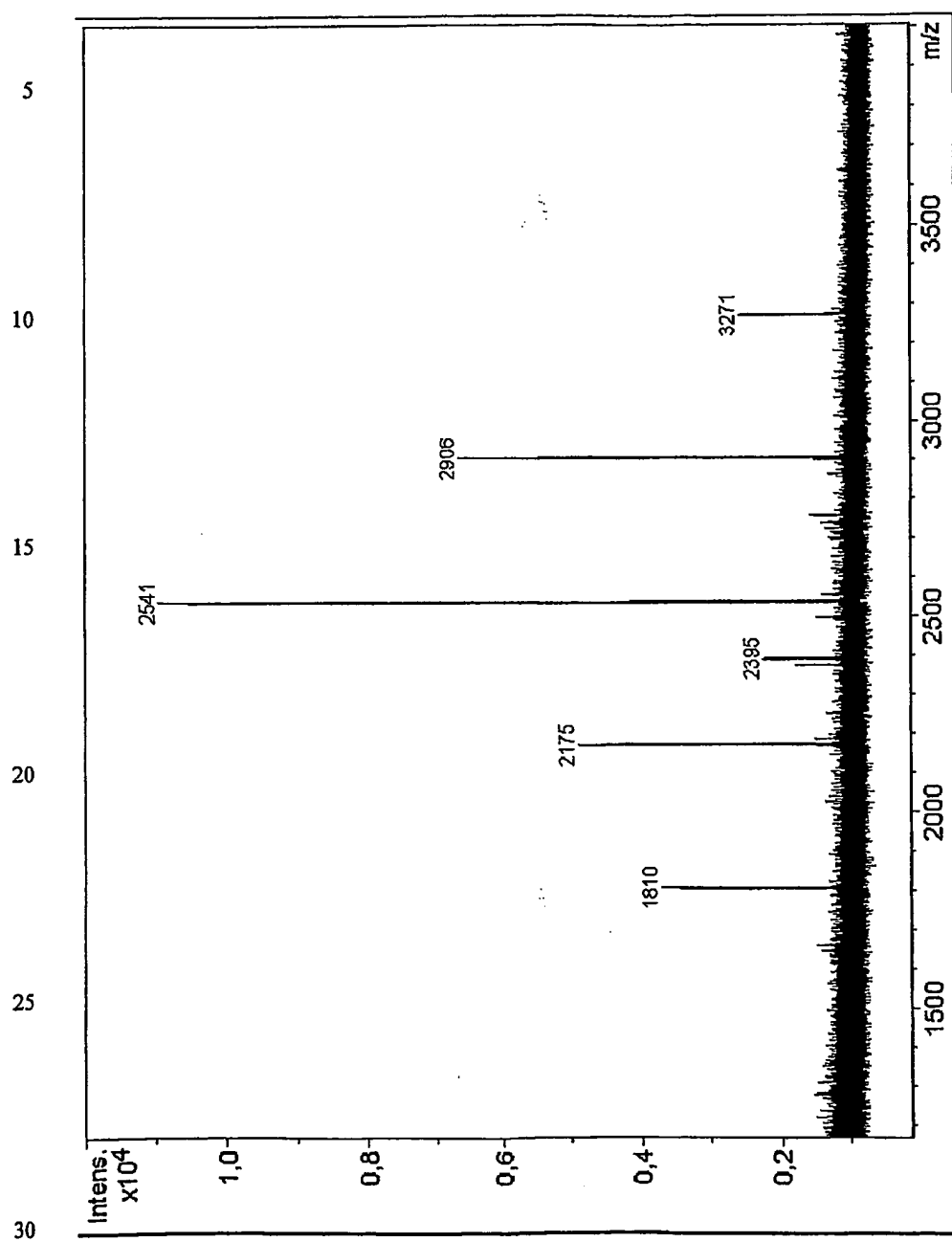


Fig. 18

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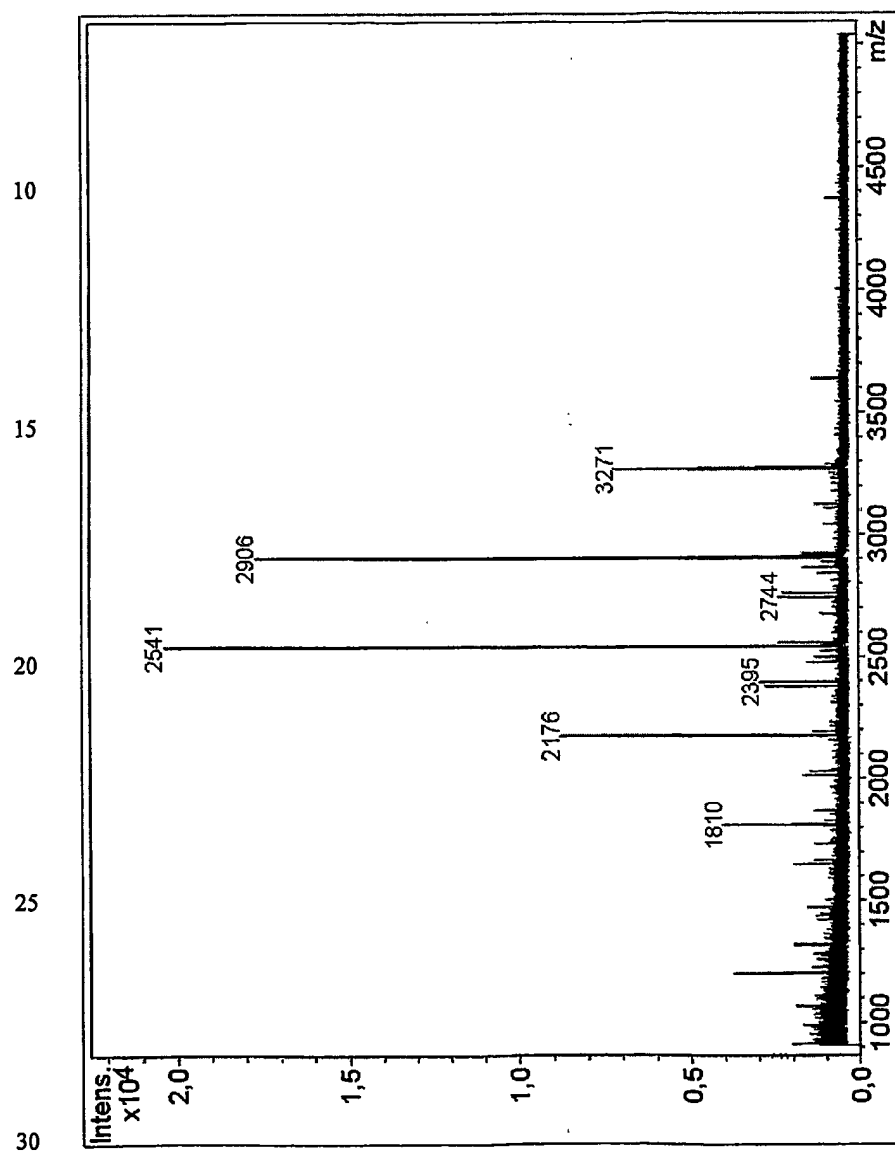


Fig. 19

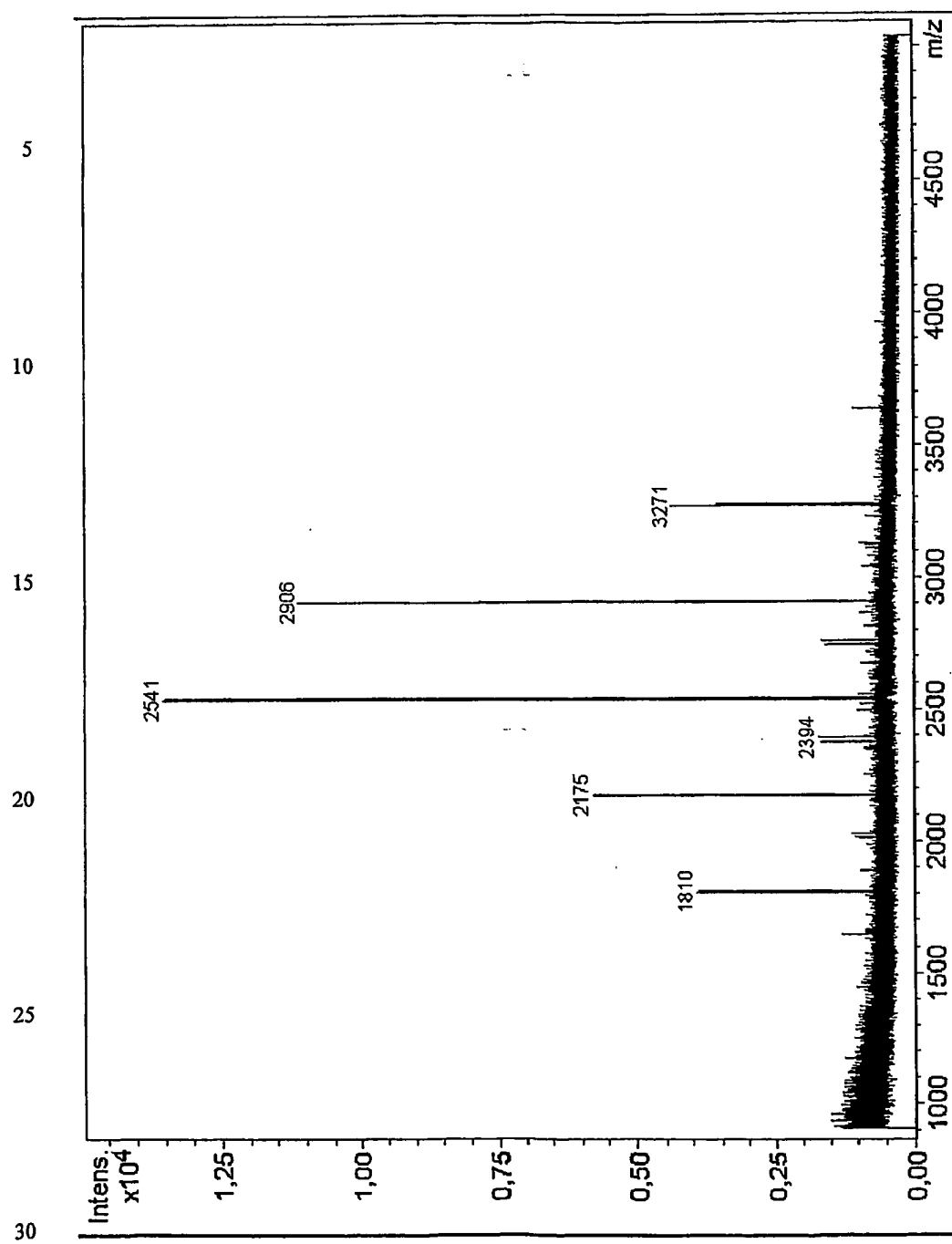


Fig. 20

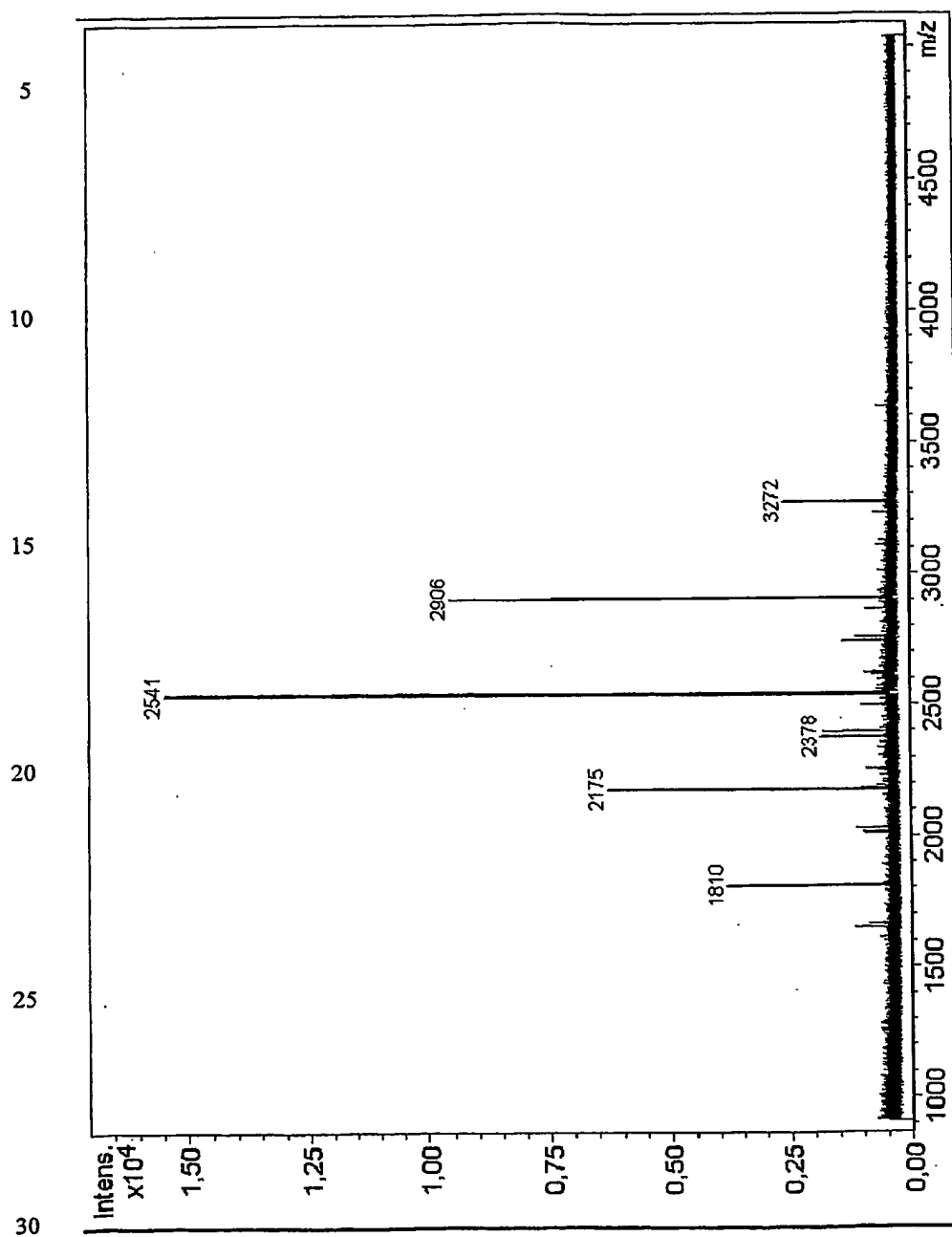
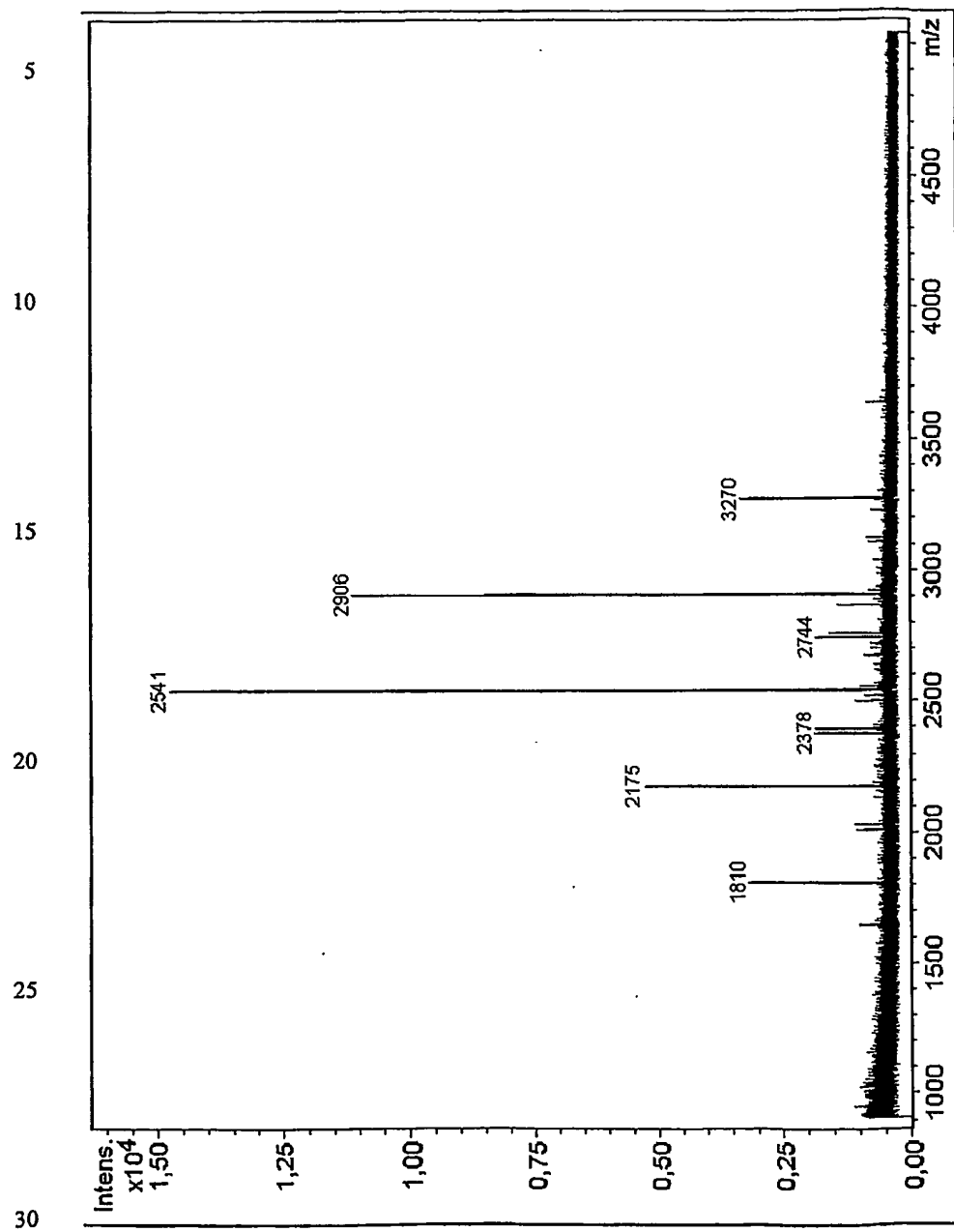


Fig. 21



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 03/08859

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 C08B31/12 A61K47/48 C07K14/505 C07K17/10

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C08B A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 02 080979 A (SOMMERMEYER KLAUS ;FRIE SVEN (DE); EICHNER WOLFRAM (DE); SCHARPF R) 17 October 2002 (2002-10-17) cited in the application example 3	1-70
E	WO 03 074087 A (HEMBERGER JUERGEN ;ORLANDO MICHELE (DE); BIOTECHNOLOGIE GES MITTEL) 12 September 2003 (2003-09-12) examples 11-15	1-70
E	WO 03 074088 A (HEMBERGER JUERGEN ;ORLANDO MICHELE (DE); BIOTECHNOLOGIE GES MITTEL) 12 September 2003 (2003-09-12) claims 1,2,4,6; examples 17-20,26	1-70
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

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- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \* & \* document member of the same patent family

Date of the actual completion of the international search

5 December 2003

Date of mailing of the international search report

18/12/2003

Name and mailing address of the ISA

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Authorized officer

Radke, M

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/08859

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	<p>-----</p> <p>LARIONOVA N I ET AL: "CONJUGATION OF THE BOWMAN-BIRK SOYBEAN PROTEINASE INHIBITOR WITH HYDROXYETHYLSTARCH" APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY, CLIFTON, NJ, US, vol. 62, no. 2/3, 1997, pages 175-182, XP001122297 ISSN: 0273-2289 page 178, paragraph 3</p> <p>-----</p> <p>A</p> <p>LEENDERS R G G ET AL: "beta-Glucuronyl Carbamate Based Pro-moieties Designed for Prodrugs in ADEPT" TETRAHEDRON LETTERS, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 36, no. 10, 6 March 1995 (1995-03-06), pages 1701-1704, XP004028569 ISSN: 0040-4039 page 1702</p> <p>-----</p>	

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